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The jigsaw of PRRSV virulence

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of the, probably, most economically important disease for the pig industry worldwide. This disease, characterised by producing reproductive failure in sows and respiratory problems in growing pigs, appeared in the late 1980s in the United States and Canada. Since its appearance, strains capable of producing higher mortality rates as well as greater severity in clinical signs and lesions than classical strains have been identified. However, since the first reports of these "virulent" PRRSV outbreaks, no homogeneity and consensus in their description have been established. Moreover, to the authors' knowledge, there is no published information related to the criteria that a PRRSV strain should fulfil to be considered as a "virulent" strain. In this review, we revise the terminology used and gather the information related to the main characteristics and differences in clinical signs, lesions, viral replication and tropism as well as immunological parameters between virulent and classical PRRSV strains and propose a first approximation to the criteria to define a virulent PRRSV strain.

1. PRRSV: from its origin to date

In the late 1980s, respiratory and reproductive failures associated with a new disease were reported in different farms in the United States and Canada. Due to the lack of a known cause, this disease received different names such as "Mystery Swine Disease" (MSD) or "Swine Infertility and Respiratory Syndrome" (SIRS) (Hill, 1990; Keffaber, 1989). It was not until 1991, when the causative agent of this disease, a virus, was isolated in Lelystad, the Netherlands (Wensvoort et al., 1991) and just one year later, another strain of the same virus, VR-2332, was isolated in the United States (Collins et al., 1992). Since then, these two strains were considered as the reference strains for each territory: Lelystad-virus strain (LV) for Europe and VR-2332 strain for North America (Collins et al., 1992; Wensvoort et al., 1991). Shortly, during the first International SIRS Symposium in Minnesota in 1992, the disease received by consensus its final and current name, Porcine Reproductive and Respiratory Syndrome (PRRS), and the aetiological agent PRRS virus (PRRSV) (Meredith, 1992).

After genomic sequencing of PRRSV open reading frames (ORF)5 and ORF7, the reference strains, LV and VR-2332, were found to share only 55–70 % of their nucleotide sequence (Collins et al., 1992; Murtaugh et al., 1995). This finding, together with the enormous antigenic heterogeneity observed between both strains (Wensvoort et al., 1992), led to the consideration of two different genotypes of the virus, European or type 1 genotype (also known as PRRSV-1) and American or type 2 genotype (also known as PRRSV-2). Furthermore, the analysis of ORF5 and ORF7 has allowed differentiating various subtypes or lineages within both PRRSV-1 and PRRSV-2 (Shi et al., 2010; Stadejek et al., 2006). Over the years, the rise in genetic differences has driven to the current classification of the virus within the new genus *Betaarterivirus* (family *Arteviridae*, order *Nidovirales*) as two distinct viral species, *Betaarterivirus suid-1* (for PRRSV-1; subgenus *Eurpobartevirus*) and *Betaarterivirus suid-2* (for PRRSV-2; subgenus *Ampobartevirus*) (Brinton et al., 2018).

2. Emergence and re-emergence of virulent PRRSV strains

The first report of a "virulent" PRRSV strain dates from 1995 in the southwest of Iowa. This PRRSV-2 strain, VR-2385, was isolated in a farm with a high number of late-term abortions and severe respiratory disease with high mortality in piglets from neonatal to growing period (Halbur et al., 1995). Between 1996 and 1997, other PRRSV-2 strains named as "atypical", such as SDSU 73, JA 142 and 17198−6, were isolated in Iowa and Oklahoma and associated with severe disease outbreaks of high sow

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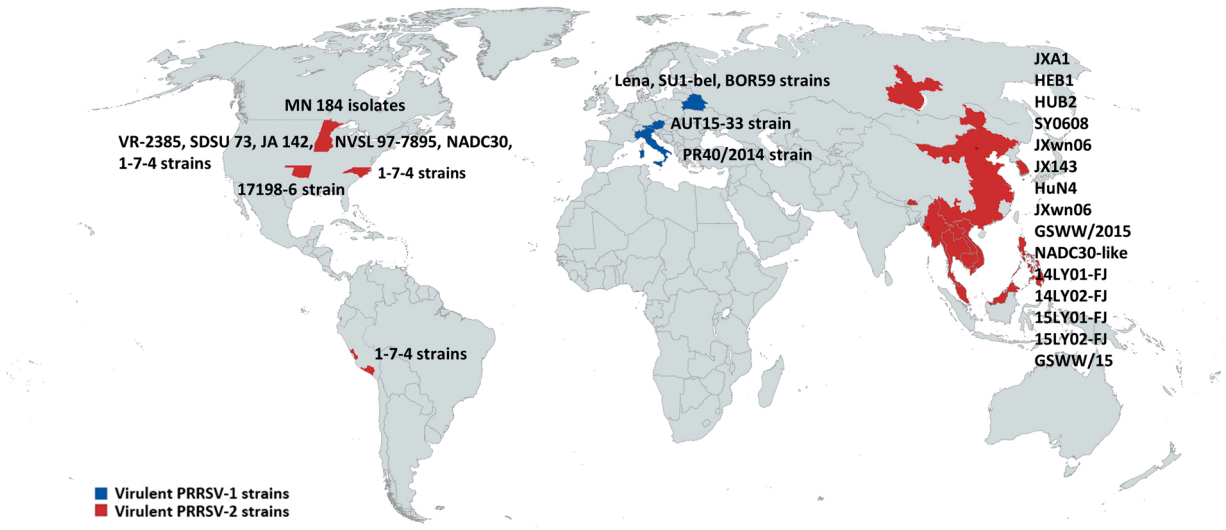

Fig. 1. Virulent PRRSV-1 (blue) and virulent PRRSV-2 (red) strains associated with field outbreaks all over the world (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

mortality and severe "abortion-storm" (Johnson et al., 2004; Mengeling et al., 1998; Roof et al., 2003). Late in 2001, many PRRSV-2 "virulent" strains of a seemingly novel PRRSV were identified in farms from southern Minnesota, named as "MN 184 isolates" (Johnson et al., 2004).

In the summer of 2006, an outbreak of a disease initially called "porcine high fever disease" was first reported in China, affecting more than 2,000,000 pigs in 10 provinces (Tian et al., 2007). The disease was characterised by high fever (40−42 °C), multifocal petechiae and ecchymosis and high mortality rate on affected farms. Molecular assays showed that "highly pathogenic" PRRSV (HP-PRRSV) was the potential aetiological agent of the epidemic, identifying several virulent strains belonging to PRRSV-2, such as JXA1, HEB1, HUB2, JX143, SY0608 and HuN4 (Li et al., 2007; Lv et al., 2008; Tian et al., 2007; Zhou et al., 2008), as causal agents. These viruses spread very fast affecting different countries in Asia, including Vietnam, Bhutan, Cambodia, Laos, Malaysia, Myanmar, Philippines, Thailand and Singapore (Feng et al., 2008; An et al., 2011). In 2008, a "highly virulent" PRRSV-2 strain, NADC30 strain, was reported in a farm from Iowa associated with severe respiratory disease (Brockmeier et al., 2012b). Curiously, several field isolates of PRRSV-2 were described in China from 2013 onwards with a high nucleotide similarity to that of NADC30 strain (93.2 % - 95.8 %), being designated as NADC30-like PRRSVs (Li et al., 2016). In 2014, additional strains (14LY01-FJ, 14LY02-FJ, 15LY01-FJ and 15LY02-FJ), resulting from the recombination of JXA1 and NADC-30-like strains, caused several outbreaks in China (Liu et al., 2017). That year, several virulent PRRSV-2 strains, called 1−7-4 strains (IA/2014/NADC34, IA/2013/ISU1 and IN/2014/ISU-5) were identified in North Carolina and Iowa (Van Geelen et al., 2018), which have been recently associated with virulent PRRSV-2 outbreaks in Peru (Ramírez et al., 2019).

Coinciding with the appearance of the first outbreak in China, a PRRSV strain named Lena, which produced high fever, reproductive problems, weak new-borns, as well as severe respiratory disorders and high mortality in growing pigs, was isolated in a Belarusian farm (Karniychuk et al., 2010). The evaluation of the clinical and lesional findings revealed similarities with the "atypical" American strains isolated in the 1990s (Johnson et al., 2004; Karniychuk et al., 2010). However, the phylogenetic analysis revealed that Lena strain differed from de North American reference strain VR-2332 and was considered as a "highly pathogenic" PRRSV-1 subtype 3 strain (Karniychuk et al., 2010). In 2010, another "pathogenic" PRRSV-1 strain, named SU1-bel strain, was isolated in Belarus (Morgan et al., 2013). Initially, the virulence of PRRSV-1 strains was associated with belonging to subtype 3, such as Lena and SU1-bel strains; however, later on, strains from subtypes 1 and 2 were found to cause fatal PRRSV-1 outbreaks. Thus, the "highly pathogenic" PRRSV-1 strains BOR59 (subtype 2), PR40/2014 (subtype 1) and AUT15−33 (subtype 1) have been sequentially reported from 2009 to 2015 in farms from Belarus, Italy and Lower Austria, respectively (Canelli et al., 2017; Sinn et al., 2016; Stadejek et al., 2017). Virulent PRRSV-1 and virulent PRRSV-2 strains associated with field outbreaks all over the world are represented in Fig. 1.

3. The complexity of the used terminology

Different terms have been used to refer to those strains causing more severe clinical manifestations and high mortality, such as "atypical", "virulent", "highly virulent", "high or highly pathogenic" or "pathogenic" strains (Table 1). Although these terms allow the identification of the strain involved in every particular outbreak, not all of them may be appropriate.

The term "atypical" is a word for "abnormal" or "unusual", in this context, it refers to some aspects beyond what would be expected for a given pathogen, but this term neither specify the process nor defines anything in particular. Pathogenicity and virulence are sometimes used in many scientific disciplines interchangeably as the ability to invade, and injure the host's tissues (Hibbs and Young, 1995). However, pathogenicity is a qualitative term which describes the ability of a pathogen to cause disease, whereas virulence is a quantitative term which defines the severity or intensity of the disease caused by the pathogen (Hibbs and Young, 1995; Thomas and Elkinton, 2004). Thus, PRRSV is pathogenic, because it is able to produce disease, in general terms, reproductive disorders in sows and respiratory disease in growing pigs, but only "virulent" or "high virulent" PRRSV strains may cause increased mortality, abortion-storms or a severe interstitial pneumonia accompanied by a strong inflammatory response and severe suppurative bronchopneumonia. Several factors, such as the ability to entry into target cells, the replication rate, the damage to host cells and the induction of cell death or specific immune response, among other factors, may determine the capability of a virus to infect the host (pathogenicity) but certainly the severity of the infection (virulence) (Hibbs and Young, 1995). Therefore, "virulent" and "high virulent" strains seem to be the correct terminology in those outbreaks characterised by "severe" clinical signs.

Due to the lack of homogeneity and consensus in the nomenclature of these strains, we focus on the main characteristics of their virulence described in field outbreaks as well as experimental trials to define the criteria that a strain should fulfil to be considered as "virulent" or "high virulent".

Table 1
Terminology used in the descriptions of the emergence of virulent PRRSV strains.

Year	Strain	Genotype	Terminology	Reference
1995	VR-2385	PRRSV-2	Virulent	Halbur et al., 1995
1996–1997	SDSU 73 17198–6	PRRSV-2	Atypical	Roof et al., 2003
1997	JA 142	PRRSV-2	Atypical or acute	Mengeling et al., 1998
1997	NVSL 97–7895	PRRSV-2	Highly virulent	Truong et al., 2004
2001	MN 184 isolates	PRRSV-2	Virulent	Johnson et al., 2004
2006	JXA1 HEB1 HUB2	PRRSV-2	Highly pathogenic	Tian et al., 2007
2006	SY0608	PRRSV-2	Highly pathogenic	Li et al., 2007
2006	JXwn06	PRRSV-2	Highly virulent	Zhou et al., 2008
2007	Lena	PRRSV-1	Highly pathogenic	Karniychuk et al., 2010
2008	JX143	PRRSV-2	Highly pathogenic	Lv et al., 2008
2008	HuN4	PRRSV-2	Highly pathogenic	Zhou et al., 2008
2008	NADC30	PRRSV-2	Highly virulent	Brockmeier et al., 2012b
2009	BOR59	PRRSV-1	Highly pathogenic	Stadejek et al., 2017
2010	SU1-bel	PRRSV-1	Pathogenic	Morgan et al., 2013
2014	PR40/2014	PRRSV-1	Highly pathogenic	Canelli et al., 2017
2014–2015	14LY01-FJ 14LY02-FJ 15LY01-FJ 15LY02-FJ	PRRSV-2	Highly pathogenic	Liu et al., 2017
2014	1–7-4	PRRSV-2	Virulent	Van Geelen et al., 2018
2015	GSWW/15	PRRSV-2	Highly virulent	Bai et al., 2018
2015	AUT15–33	PRRSV-1	Virulent	Sinn et al., 2016

4. Correlates of virulence

4.1. Viral genetic determinants of virulence

Since the re-emergence of virulent strains, numerous studies have described the presence of discontinuous 30 amino acids deletions in the non-structural protein 2 (Nsp2)-coding region (ORF1a) in both virulent PRRSV-1 and PRRSV-2 strains, considering these deletions as genetic markers of virulent strains (Canelli et al., 2017; Do et al., 2016; Li et al., 2007; Tian et al., 2007; Zhou et al., 2008). However, the finding of these mutations also in low virulent strains hampers demonstrating the relationship between Nsp2 deletions and the virulence of the strains (Li et al., 2010; Zhou et al., 2014), remaining its biological significance still unclear. Thus, whereas deletion of specific Nsp2 epitopes may play a role in modulating host immunity (Chen et al., 2010), a recent study has shown a loss of infectivity in a mutant JXwn06 Nsp2, suggesting indeed a role in the cellular tropism (Song et al., 2019). Furthermore, findings of Li et al. (2014) also suggested that ORF1b, specifically Nsp9 and Nsp10, contributes to the fatal *in vivo* and *in vitro* virulence of the same PRRSV-2 strain (JXwn06), with the residues 586 and 592 of Nsp9 being highlighted as the critical sites regulating the replication of this virulent PRRSV-2 strain (Xu et al., 2018). In this line, other researchers recently identified a role of the amino acids at positions 519 and 544 in Nsp9 in the pathogenicity and replication efficiency of the virulent HuN4 PRRSV-2 strain (Zhao et al., 2018). These previous results are somehow in agreement with a previous study with a virulent PRRSV infectious clone (FL12) from the virulent NVSL 97–7895 PRRSV-2 strain performed by Kwon et al. (2008) who pointed out that Nsp3–8 (ORF1a) and ORF5 regions were the location of the main virulence determinants, together with Nsp1–3 (ORF1a), Nsp10–12 (ORF1b) and ORF2, suggesting that PRRSV virulence determinants are multigenic. This hypothesis would be also supported by the finding of mutations in the ORF5 resulting in amino acids deletions as possible viral genetic determinants of virulence in some virulent Asian PRRSV-2 strains, such as HuN4 (Do et al., 2016; Ko Ko et al., 2019; Zhou et al., 2008). Hence, next generation sequencing studies comprising a wide range of PRRSV strains are required to further confirm genetic differences between different virulent strains, with a big effort being required for virulent PRRSV-1 strains according to the scarce availability of data, and between virulent and low virulent strains.

4.2. Clinical signs, temperature and lesions

One of the main features of PRRS is the wide variability in clinical signs, ranging from asymptomatic animals to a devastating disease in the case of virulent PRRSV strains (Karniychuk et al., 2010; Zhou et al., 2008). High morbidity and mortality, elevated fever, higher viraemia and co-infections with other bacteria are common findings to virulent strains infections.

In the outbreaks that took place in China, about two million pigs were affected, dying roughly 20 % of them (Tian et al., 2007). Other authors reported a morbidity rate from 50 to 100 % with a high proportion of deaths in pigs of all ages (20–100 %) (Li et al., 2007; Liu et al., 2017; Zhou et al., 2008). In the outbreak caused by PRRSV-1 Lena strain in Belarus, a death rate of 70 % in pre-weaning and growing pigs was recorded (Karniychuk et al., 2010). Mortality rates around 50 % were declared in the outbreaks of Italy and Austria, affecting mainly new-born and weaner piglets (Canelli et al., 2017; Sinn et al., 2016). Given the collected data from field outbreaks, a mortality rate of 20 % or higher in pigs from different groups of age might be expected when a virulent PRRSV strain is present on a farm and different from classical PRRSV, where growing pigs are mainly affected. Although mortality rate of virulent PRRSV strains is difficult to be reproduced in experimental conditions, some trials with virulent PRRSV-1 and PRRSV-2 strains succeed on it. For instance, in an experimental study performed by Johnson et al. (2004) with nine different PRRSV-2 strains differing on their virulence, only virulent strains caused mortality during the second and third week after infection (JA 142, 10 %; SDSU 73, 20 %; and MN 184, 50 % of mortality, with a total mortality of 22 %). In another experiment performed with the virulent GSWW/15 PRRSV-2 strain, isolated in China in 2015, 14 out of 24 pigs gradually died from 35 to 118 days post-infection (dpi) (Bai et al., 2018). Similar outcomes were described in the experimental study carried out with the virulent Lena PRRSV-1 strain by Karniychuk et al. (2010). However, other experimental studies with virulent PRRSV-1 strains reported no mortality along the trial (Canelli et al., 2017; Morgan et al., 2013; Rodríguez-Gómez et al., 2019; Stadejek et al., 2017). The biocontainment environment in which these trials are carried out differing with field conditions. Thus, the secondary bacterial infections and other viral pathogens have been a common and evident issue associated with virulent PRRSV-1 and PRRSV-2 field outbreaks which could play a synergetic role on virulent PRRSV pathogenesis. In this way, bacterial secondary infections, could explain these differences in mortality rates

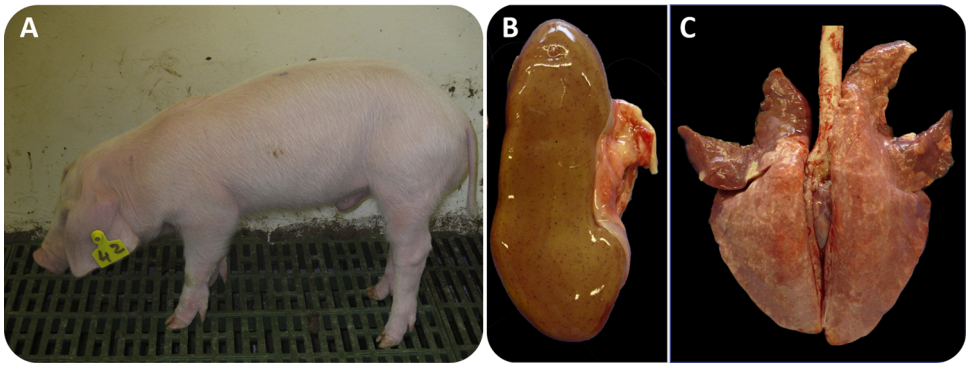

Fig. 2. Piglet of 4 weeks of age, intranasally infected with the virulent PRRSV-1 Lena strain (10^5 $TCID_{50}$), showing evident respiratory distress (**A**). Kidney (**B**) and lung (**C**) from a four-week-old pig, intranasally infected with Lena and euthanised at 6 dpi, exhibiting petechiae in the cortical surface and cranioventral consolidation, respectively.

among experimental studies and field outbreaks. Moreover, the age of animals and the short duration of the experiment, limited to the first 2–3 weeks post-infection, may also give an explanation.

The high fever induced by virulent PRRSV strains is a consistent finding in both clinical cases and experimental trials, ranging from 40.5–42 °C, starting as early as 2 dpi and being maintained from 1 up to 4 weeks (Brockmeier et al., 2012b; Canelli et al., 2017; Halbur et al., 1995; Karniychuk et al., 2010; Li et al., 2017; Liu et al., 2017; Lv et al., 2008; Morgan et al., 2013; Renson et al., 2017; Rodríguez-Gómez et al., 2019; Sinn et al., 2016; Stadejek et al., 2017; Van Geelen et al., 2018; Weesendorp et al., 2014). Other commonly observed clinical signs in pigs infected with virulent strains are anorexia, lethargy, conjunctivitis, petechiae and erythematous blanching rash in skin, cyanosis, diarrhoea and evident respiratory disease (Fig. 2A), which negatively impacts on the sanitary status of the pigs and on the average daily weight gain (ADWG) (Canelli et al., 2017; Halbur et al., 1995; Li et al., 2017; Morgan et al., 2013; Rodríguez-Gómez et al., 2019; Sinn et al., 2016; Stadejek et al., 2017; Zhou et al., 2008).

Besides clinical signs, the development and extent of the lesions are directly connected with the virulence of PRRSV. Lesions are mostly restricted to lung in infections with low virulent strains, and, if uncomplicated, go unnoticed (Collins et al., 1992; Wensvoort et al., 1991). However, gross lesions caused by virulent PRRSV strains are characterised by severe diffuse interstitial pneumonia which usually is accompanied by suppurative bronchopneumonia, pleurisy, haemorrhagic spots in lung, kidney (Fig. 2B) and lymph nodes, thymus atrophy, intestine ulceration, serous fluid in the thoracic cavity and lymphadenopathy (Brockmeier et al., 2012b; Canelli et al., 2017; Halbur et al., 1995; Li et al., 2016, 2017; Liu et al., 2017; Rodríguez-Gómez et al., 2019; Roof et al., 2003; Stadejek et al., 2017; Tian et al., 2007; Wang et al., 2011). Roof et al. (2003) reported that the PRRSV-2 SDSU 73 strain caused an average of 70 % gross lung lesions whereas the reference PRRSV-2 strain VR-2332 induced an average of 31 %. Foci of consolidation, mainly in the cranioventral portions of the lungs (Fig. 2C), together with fibrinous pleuropneumonia have been well described in cases of virulent PRRSV strains (Brockmeier et al., 2012b; Karniychuk et al., 2010; Li et al., 2016; Rodríguez-Gómez et al., 2019; Sinn et al., 2016; Zhou et al., 2008). These findings together with the severity of the clinical signs are probably not only due to the direct effect of virulent PRRSV, but also to the proliferation and growth of specific commensal pathogens from the lung microbiome as well as to secondary bacterial infections. In fact, bacteria such as *Streptococcus suis*, *Streptococcus hyicus* and *Trueperella pyogenes* have been isolated in pigs after infection with virulent PRRSV-1 and PRRSV-2 strains (Canelli et al., 2017; Johnson et al., 2004; Karniychuk et al., 2010; Sinn et al., 2016).

4.3. *Expanded tropism (I): wider tissue distribution and higher viral load*

Comparative studies between virulent and low virulent strains have shown a greater number of PRRSV positive cells in various tissues in favour of the former (Amarilla et al., 2015, 2016; Halbur et al., 1996; Li et al., 2012; Rodríguez-Gómez et al., 2019; Ruedas-Torres et al., 2020; Sánchez-Carvajal et al., 2020; Weesendorp et al., 2014). Halbur et al. (1996) demonstrated by immunohistochemistry the presence of PRRSV antigen in a high number of cells from lung, heart, lymph node, thymus, spleen, liver, kidney and intestine from pigs infected with the virulent PRRSV-2 VR-2385 strain, compared to those infected with the reference PRRSV-1 LV strain. With the re-emergence of virulent strains, viral antigen has been detected in a multitude of tissues, such as brain, liver, heart, kidney, cerebellum, stomach, small and large intestines and even hypodermis (Li et al., 2012; Lv et al., 2008; Tian et al., 2007). In addition, significant differences have been also detected regarding viral load in target organs between virulent and low virulent PRRSV strains (Han et al., 2015; He et al., 2012; Hu et al., 2013; Liu et al., 2010; Ruedas-Torres et al., 2020; Sánchez-Carvajal et al., 2020). In general, virulent strains yield a higher viral load than low virulent strains. This is the case of the virulent PRRSV-1 Lena strain and the virulent PRRSV-2 JXwn06 strains, giving rise to a viral load of around 10^7 $TCID_{50}$/mL or 10^7 copies/mL in the lung and lymphoid organs, respectively, much higher than reference or low virulent strains (Guo et al., 2013; Karniychuk et al., 2010; Ruedas-Torres et al., 2020; Sánchez-Carvajal et al., 2020). The PRRSV-2 HuN4 has been demonstrated to produce higher viral load in lungs (with values around 10^{10-12} copies/mL) and lymphoid organs (with values around 10^{8-12} copies/mL) (Han et al., 2015; He et al., 2012; Hu et al., 2013; Liu *et al.,* 2010; Wang et al., 2014), approximately $10^{3\text{-}4}$ copies/mL higher than the low virulent CH1a strain (Han et al., 2015; He et al., 2012; Wang et al., 2014), and maintaining high viral load (10^7 copies/mL) until 30 dpi, not only in lung and lymphoid tissue but also in kidney, liver, heart, stomach and brain (Hu et al., 2013; Liu et al., 2010; Wang et al., 2011).

Similarly, viraemia has been reported to be higher in virulent strains with differences around $10^{1.5-2}$ $TCID_{50}$/mL or 10^2copies/mL between pigs infected with either virulent PRRSV-1 strains, such as Lena, BOR59 and AUT15−33 strains, or virulent PRRSV-2 strains, such as JA 142, SDSU 73, MN 184, 17198−6, NADC30, HuN4 and JXwn06 strains, compared with various low virulent strains (Brockmeier et al., 2012b; Guo et al., 2013; Johnson et al., 2004; Karniychuk et al., 2010; Ruedas-Torres et al., 2020; Sánchez-Carvajal et al., 2020; Stadejek et al., 2017; Weesendorp et al., 2013a, 2013b; 2016). However, in the case of SU1-bel strain, lower and shorter viraemia during the whole study in comparison with the reference PRRSV-1 LV strain was detected. In this case, SU1-bel-infected pigs presented a greater clinical score and lung gross pathology, which together with immunological aspects were considered as correlates of virulence for the SU1-bel strain instead of

viraemia (Morgan et al., 2013).

4.4. Expanded tropism (II): putative alternative receptors for virulent PRRSV strains

At least seven cellular molecules have been suggested so far as receptors for PRRSV, including heparan sulfate, vimentin, CD151, sialoadhesin (Sn, CD169 or siglec-1), siglec-10, dendritic cell-specific intercellular adhesion molecule-3-grabbing non integrin (DC-SIGN) and CD163 (Van Breedam et al., 2010; Xie et al., 2017). Although Sn have been proved to play an important role in PRRSV attachment, entry, and endocytosis, CD163 is the only essential receptor required for PRRSV infection (Burkard et al., 2018; Whitworth et al., 2016). Accordingly, several studies have tried to elucidate if virulent PRRSV strains get advantage of alternative receptors to enhance its viral entry. Thus, it has been reported that virulent PRRSV-1 Lena strain was able to infect $Sn^{-}CD163^{-}$ cells in a nasal mucosa explant system (Frydas et al., 2013). On the other hand, experiments with cell lines demonstrated that siglec-10 was able to replace the role of Sn after the infection with the virulent PRRSV-2 MN 184 strain on a CD163-tranfected cell line (Xie et al., 2017). In this context, the same PRRSV strain can infect either through siglec-1 or siglec-10 but the rate of infection may change, which might be linked with the virulence of a given strain (Xie et al., 2018). Although it has been speculated that certain virulent PRRSV strains could use a wide range of receptors that would allow them to replicate more easily in target cells, as well as in a variety of cell types, it is still not clear if they do play a cornerstone in PRRSV virulence. Further studies are needed to establish the true role of the different receptors during virulent PRRSV infection *in vivo*, taking into account the study of target organs, such as the lung and primary lymphoid organs, and comparing results with well characterised strains.

4.5. Immunological aspects of virulent PRRSV strains

4.5.1. Innate immune response: cell subsets and inflammatory response

The role of virulent PRRSV strains on different innate immune cell subsets is still controversial. It has been reported that high virulent PRRSV-1 Lena strain is able to diminish the frequency of $\gamma\delta$ T cells in infected pigs when compared to reference PRRSV-1 LV strain (Weesendorp et al., 2013a), however, this outcome was not detected in other virulent PRRSV-1 strains such as SU1-bel or PR40/2014 (Ferrari et al., 2018; Morgan et al., 2013). This is also the case for the frequency of NK cells, which remained invariable after infection with the virulent PRRSV-1 Lena and SU1-bel strains (Morgan et al., 2013; Weesendorp et al., 2013a), whereas a significant increase together with a decrease in the frequencies of $CD14^{+}CD16^{+}$ and $CD14^{+}CD163^{+}$ subsets were observed in PR40/2014-infected pigs, considered as a hallmark for this particular virulent strain (Canelli et al., 2017; Ferrari et al., 2018).

In regard to cytokine production, virulent PRRSV strains have demonstrated to trigger a characteristic inflammatory cytokine cascade, particularly mediated by interleukin 1 alpha (IL-1α) and IL-1β but also interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α), accompanied by evident clinical signs, fever and severe tissue damage, especially in the lung (Amarilla et al., 2015; Li et al., 2017; Liu et al., 2010; Renson et al., 2017; Sánchez-Carvajal et al., 2020; Weesendorp et al., 2013a). Low virulent PRRSV has been described for its ability to inhibit type I interferon (IFN) production (Albina et al., 1998), finding which has been also demonstrated during *in vitro* studies with some virulent PRRSV-1 and PRRSV-2 strains, such as Lena, SDSU 73, MN 184, SY0608, JXwn06 (Baumann et al., 2013; Brockmeier et al., 2012a; García-Nicolás et al., 2014; Huang et al., 2014). However, in *in vivo* studies, an increase in the level of IFN-α parallel to an increase in PRRSV viral load has been observed (Li et al., 2017; Liu et al., 2010; Renson et al., 2017; Weesendorp et al., 2013a). This fact points out that IFN-α might not be effective enough in controlling virulent PRRSV replication, and thus, the role of this cytokine during the pathogenesis of virulent PRRSV infection needs further revision.

Parallel to the inflammatory cytokine cascade, an anti-inflammatory response is also triggered during the infection with certain virulent PRRSV strains. In *in vivo* studies performed with the virulent PRRSV-2 HuN4, JXwn06, HV-PRRSV and NADC30 strains, an increase in the serum concentration of IL-10 was detected (Brockmeier et al., 2012b; Guo et al., 2013; Han et al., 2015; Li et al., 2017; Wang et al., 2011). Furthermore, an increase in the number of $Foxp3^{+}$ cells as well as $CD200R^{+}$ cells has been observed in the lung of virulent PRRSV-1 Lena strain infected piglets (Sánchez-Carvajal et al., 2020). The increase in all these molecules most probably answers to an attempt to control the tissue damage caused by inflammation, but they can also lead to a weakened cell-mediated immunity in more advanced stages of the infection.

4.5.2. Adaptive immune response: cellular and humoral immune responses

Virulent PRRSV-1 (Lena) and PRRSV-2 (JXwn06, VR-2385 and SH-PRRS01) strains influence the host adaptive immune response by, among other actions, modulating surface molecules involved in antigen presentation such as swine leukocyte antigen (SLA) class I (SLA-I) and class II (SLA-II) (Cao et al., 2016; Du et al., 2016; Qi et al., 2017; Weesendorp et al., 2013b). The impairment in the surface expression of SLA-I and SLA-II points out to a potential mechanism involved in the evasion of antigen recognition and activation of cellular and humoral immune responses.

IFN-γ plays a key role in the induction of cellular immunity. Whereas IFN-γ secreting cells (IFN-γ-SC) remained at low levels during the time frame of infection in Lena-infected pigs suggesting a poor induction of cell-mediated immune response (Weesendorp et al., 2013b); other virulent PRRSV-1 strains, such as PR40/2014 and SU1-bel, displayed an increase in the level of IFN-γ-SC when compared with low virulent strains (Ferrari et al., 2018; Morgan et al., 2013). This finding has been hypothesised to contribute to the apparent faster clearance observed for SU1-bel-infected pigs (Morgan et al., 2013), however, it was not the case for the virulent PRRSV-1 PR40/2014 strain (Canelli et al., 2017). Besides, a peak of serum and lung lavage IFN-γ concentration in virulent PRRSV-1- and PRRSV-2-infected pigs (Ferrari et al., 2018; Guo et al., 2013; Li et al., 2017; Liu et al., 2010; Renson et al., 2017; Sánchez-Carvajal et al., 2020; Wang et al., 2011) has been associated with an aggravation of the inflammatory response, which would explain the severity in the clinical signs and gross lesions (Brockmeier et al., 2012b; Liu et al., 2010; Morgan et al., 2013). The increase in the level of IFN-γ has been also linked with an increase in the number of memory CD4 T cells ($CD3^{+}CD4^{+}CD8^{+}$) in pigs infected with the virulent PRRSV-1 SU1-bel and PR40/2014 strains and with the virulent PRRSV-2 HV-PRRSV strain in comparison with low virulent strains (Ferrari et al., 2018; Li et al., 2017; Morgan et al., 2013). In the same way, a similar increase has been observed in the level of cytotoxic T lymphocytes ($CD3^{+}CD4^{-}CD8\alpha^{+}CD8\beta^{+}$) (Ferrari et al., 2018; Li et al., 2017; Morgan et al., 2013), which could point out to an attempt of the host to control viral replication (Morgan et al., 2013).

Controversial results have been found regarding the role of regulatory T cells (Treg's) during the course of virulent PRRSV infections. Whereas *in vitro* experiments demonstrated that certain epitopes from the N protein of the virulent PRRSV-2 BB0907 strain may comparatively induce significantly more Treg's (Fan et al., 2015), *in vivo* studies have shown either similar or even a lower frequency of Treg's in virulent PRRSV-1 SU-1 bel and PR40/2014 strains, respectively, with respect to the reference PRRSV-1 strain (Ferrari et al., 2018; Morgan et al., 2013).

Regarding the humoral immune response, an early B cell lymphopenia, linked with a reduction in the frequency of $SWC1^{-}CD172a^{-}SWC8^{high}$ cells, have been observed in virulent PRRSV-1 infected pigs (Ferrari et al., 2018; Morgan et al., 2013; Weesendorp et al., 2013a), which could be associated with the mobilisation of these cells to lymphoid organs where polyclonal activation takes places (Weesendorp et al., 2013a). Nevertheless, the concentration of

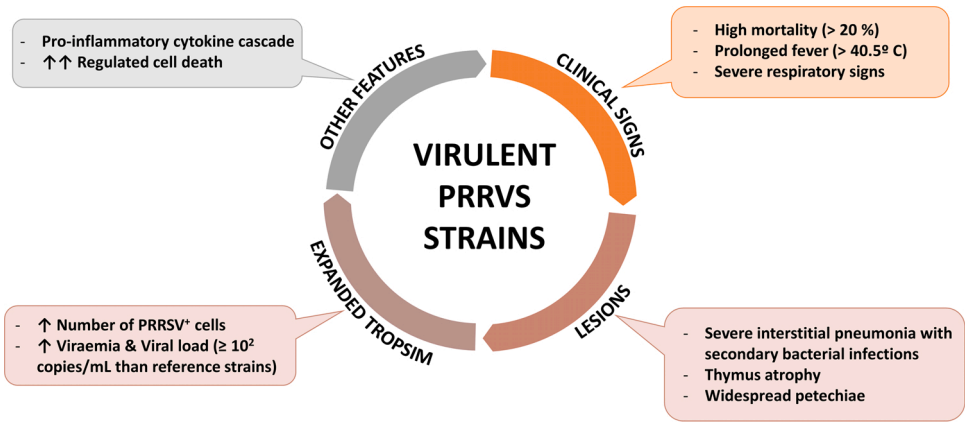

Fig. 3. Summary of the virulence correlates to define a virulent PRRSV strain.

non-neutralising PRRSV antibodies is variable among virulent *vs* low virulent strains (Brockmeier et al., 2012b; Canelli et al., 2017; Johnson et al., 2004; Li et al., 2017; Morgan et al., 2013; Sánchez-Carvajal et al., 2020; Weesendorp et al., 2013a, 2016), with earlier and higher antibody response being associated with the magnitude of the viraemia (Johnson et al., 2004). Virus neutralising (VN) antibodies have been less studied in virulent PRRSV strains and the results are also diverse. Whereas virulent PRRSV-1 PR40/2014 strain displayed a lower titre of VN antibodies in comparison with a low virulent strain (Canelli et al., 2017), the virulent PRRSV-1 Lena strain induced an earlier rise of VN antibodies, which was hypothesised to help in clearing the primary infection (Weesendorp et al., 2016).

Besides the induction of a pro-inflammatory cascade, the lack of homogeneity in the outcomes of the immune response observed in pigs infected with virulent PRRSV strains makes particularly difficult to use this criterion to categorise a strain as a virulent one.

4.5.3. Regulated cell death and virulent PRRSV strains

Regulated cell death, specifically apoptosis, is another phenomenon that has been widely studied in the context of virulent PRRSV strains, reporting high apoptosis rates in a virulence-dependent fashion associated with the release of pro-apoptotic cytokines, such as IL-1β and TNF-α (Amarilla et al., 2016; He et al., 2012; Renson et al., 2017; Ruedas-Torres et al., 2020; Wang et al., 2014). Cell death, induced by virulent PRRSV strains in lymphoid organs and lung, helps to explain the severity of the lesions, clinical signs and secondary bacterial infections observed in virulent-infected pigs (Amarilla et al., 2016; He et al., 2012; Ruedas-Torres et al., 2020; Wang et al., 2014; Sánchez-Carvajal et al., 2021). Thus, upon infection with virulent strains, thymus is one of the most severely affected, resulting in its atrophy and leading to a reduction in the total number of mature T cells (Amarilla et al., 2016; He et al., 2012; Ruedas-Torres et al., 2020). Moreover, at lung level, virulent strains lead to a marked depletion of PAMs impairing the clearance of bacterial infections (Sánchez-Carvajal et al., 2021). Exceptionally, in a study performed with pigs infected with the virulent Italian strain PR40/2014 in comparison with pigs infected with a low virulent strain (PR11/2014), no differences in the thymus atrophy and apoptosis phenomenon were found between both infected groups (Ogno et al., 2019). This finding, as well as other parameters above mentioned, shows the lack of homogeneity among some results from different virulent PRRSV strains and highlight the necessity of establishing homogeneous criteria for the classification of strains according to their virulence.

5. PRRSV virulence classification criteria

To the authors' knowledge, there is no published information related to the criteria that a PRRSV strain has to fulfil to be considered as a virulent strain. Health status of the herd will undoubtedly have an impact on mortality, exhibited clinical signs, lesions, dissemination and clearance of the virus in infected pigs; however, when performing experimental studies, it is required to include a well-characterised strain, such as Lena for PRRSV-1 and NADC30 or MN 184 for PRRSV-2, together with reference strains, LV and VR-2332 strains, respectively, to generate conclusive results.

The terms virulent and highly virulent are not well differentiated in the literature and it is difficult to establish differences. Nevertheless, during the preparation of this work, some differences regarding the elevated viraemia and tissue tropism produced by the virulent PRRSV-2 HuN4 strain could be interpreted as correlates of high virulence for this strain compared with other virulent PRRSV-2 and PRRSV-1 strains. Similarly, apart from the above-mentioned differences for this particular PRRSV-2 strain, no significant differences were found between virulent PRRSV-1 and PRRSV-2 strains. Therefore, the proposed criteria, which come next, are equally valid for both viruses (Fig. 3).

1) High mortality (20 % or higher) and morbidity affecting various age groups on the farm.
2) Severe clinical signs, including hyperthermia (40.5–42 °C), lethargy, anorexia, anaemia, cyanosis and skin lesions, diarrhoea, and more evident respiratory clinical signs (such as dyspnoea, tachypnoea and cough), which contribute to a high clinical score and a drop in the ADWG in affected animals.
3) Severe extensive interstitial pneumonia associated with an inflammatory cytokine cascade (*i.e.* IL-1, IL-6, TNF-α, IFN-γ) and usually accompanied by suppurative or fibrinous bronchopneumonia, which would indicate the participation of opportunistic and secondary bacteria.
4) Lesions in other organs, such as thymus atrophy and lymphadenopathy, vascular lesions in different organs, such as petechiae in kidneys, lymph nodes and lung, and serous fluid in thoracic cavity.
5) Higher and expanded tropism than low virulent or reference strains. Higher number of antigen-specific positive cells in lung and other tissues, mainly in lymphoid tissues, such as thymus, lymph node and tonsil, than the reference strain should be demonstrated by immunohistochemistry or *in situ* hybridisation. Higher viraemia and viral load in these organs also would be indicative of a virulent strain; differences of 10^2 copies/mL between the virulent and the reference strain should be found in serum, lung, lymph node, tonsil or thymus in virulent PRRSV-infected pigs.
6) Higher ability to induce regulated cell death in lungs and lymphoid tissues, mainly in the thymus but also in other lymphoid tissues, such as lymph nodes or tonsil.

Under field conditions, when a virulent PRRSV strain is present, we consider that at least the first four criteria (mortality up to 20 %, severe clinical signs and lesions in lung and other organs) should be evidenced.

However, although these criteria are highly correlated among them, during experimental studies it is possible that a given strain does not fulfil all of them although still behaving as a virulent strain. For that reason, we consider that a PRRSV strain should comply with at least five out of these six criteria in experimental trials to be considered as a virulent strain.

This review aims to represent the pillars to the terminology and criteria to define a virulent PRRSV strain, helping also in the classification of the virulence of other respiratory pathogens. Differences between each published experimental setting at the present time makes difficult to establish clearer criteria, thus more comparative studies are needed to give light to the jigsaw of PRRSV virulence.

Declaration of Competing Interest

The authors declare that they have no competing interest.

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