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Review article

PRRSV, the virus

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Abstract – Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-strand RNA virus that belongs to the *Arteriviridae* family. PRRSV grows in primary alveolar macrophages and in monkey kidney cell lines. The genomic RNA is approximately 15 kb. The genome encodes the RNA replicase (ORF1a and ORF1b), the glycoproteins GP_2 to GP_5, the integral membrane protein M, and the nucleocapsid protein N (ORFs 2 to 7). A comparison of nucleotide sequences of different strains indicates that European and North American strains represent two distinct antigenic types. Various PRRSV-specific monoclonal antibodies and recombinant structural proteins have been produced. Well-defined PRRSV mutants can be generated with the recently developed infectious cDNA clone of PRRSV.

PRRSV / genome organisation / structural protein / infectious cDNA clone

Résumé – Syndrome dysgénésique et respiratoire porcin, le virus. Le virus du syndrome dysgénésique et respiratoire porcin (PRRSV) est un virus à ARN simple brin de polarité positive qui appartient à la famille des *Arteriviridae*. PRRSV se multiplie sur des macrophages alvéolaires et sur des lignées cellulaires dérivées de rein de singe. L'ARN génomique est d'une longueur approximative de 15 kb. Le génome code pour la réplicase de l'ARN (ORF1a et ORF1b), les glycoprotéines GP_2 à GP_5, la protéine membranaire intégrale M, et la protéine de nucléocapside N (ORFs 2 à 7). La comparaison des séquences nucléotidiques des différentes souches indique que les souches européenne et nord-américaine représentent deux types antigéniques distincts. Divers anticorps monoclonaux spécifiques du PRRSV, et diverses protéines de structure recombinantes ont été obtenus. Des mutants bien définis du PRRSV peuvent être produits grâce au clone d'ADNc infectieux du PRRSV qui a été récemment construit.

PRRSV / organisation génomique / protéine de structure / clone d'ADNc infectieux

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1. INTRODUCTION

A new virus causing abortions in sows and respiratory problems in piglets was isolated in 1991 in the Netherlands [52]. A virus causing similar clinical symptoms was isolated in the United States in 1992 [6]. The virus was initially designated Lelystad virus (LV) but is now generally known as the porcine reproductive and respiratory syndrome virus (PRRSV). It is a small enveloped positive-strand RNA virus that together with the equine arteritis virus (EAV), lactate dehydrogenase-elevating virus, and simian hemorrhagic fever virus belongs to the *Arteriviridae* family [45]. The new *Arteriviridae* family was combined with the *Coronaviridae* family to form the newly established *Nidovirales* order. Since the discovery of PRRSV, the understanding of the virus and its disease has increased rapidly. The purpose of this paper is to briefly summarise our current knowledge of the virus. Emphasis will be made on recent information concerning virus replication, genome organisation, and viral proteins.

2. IN VITRO GROWTH CHARACTERISTICS OF PRRSV

PRRSV has a very restricted host specificity. PRRSV grows primarily in porcine alveolar lung macrophages and in macrophages of other tissues [42]. It was also recently shown to replicate in testicular germ cells such as spermatids and spermatocytes in infected boars [49]. PRRSV can grow in vitro in primary cultures of alveolar lung macrophages and in African green monkey kidney cells or derivatives thereof (CL2621 or MARC-145 cells: [5, 18]). Upon transfection of genomic RNA, PRRSV can replicate in several cell lines that cannot be infected by virus particles [35]. This finding indicates that cell tropism is determined by the presence or absence of an as yet unidentified receptor on the cell surface. Recently, monoclonal antibodies were produced that specifically bind to macrophages and prevent these cells from becoming infected by PRRSV [13]. These MAbs recognise a 210-kDa membrane protein, that might function as a putative receptor for PRRSV.

PRRSV is thought to enter the host via the standard endocytotic route. Electron microscopy has revealed PRRSV particles present in small vesicles, which appear to be clathrin-coated pits [19]. Between 3-6 h after infection, double membrane vesicles are formed [42]. This is a general feature of an arterivirus infection. It was recently shown for EAV that these double membrane vesicles are derived from the endoplasmic reticulum (ER) and carry the replication complex [40]. PRRSV is assembled when preformed nucleocapsids bud into the lumen of the smooth endoplasmic reticulum or Golgi region or both. After budding, virions accumulate in vesicles, which move to

the plasma membrane where they fuse to release the virus. In one-step growth experiments, the maximum release of PRRSV particles is between 10-20 h, the maximum titers in cell culture are $10^{6.5}$-$10^{7.5}$ $TCID_{50}$/mL. The cytopathic effect of PRRSV in macrophages and cell lines is characterised by rounding of the cells and detachment from the culture plate surface.

3. GENOME ORGANISATION

The complete genome of the LV strain of PRRSV has been sequenced [8] and has a similar organisation as other arteriviruses [45]. It is 15 kb in length and contains eight ORFs (Fig. 1A). The ORFs 1a and 1b comprise about 80% of the genome and encode the RNA-dependent RNA polymerase also known as RNA replicase. The ORF1a and ORF1ab encoded polyproteins are processed into smaller protein products, designated as nonstructural proteins (nsp). For PRRSV, only the first two N-terminal cleavage products, nsp1α and nsp1β, have been identified and have been shown to be papain-like cysteine proteases [9]. As with equine arteritis virus, another cysteine protease (nsp2) and a serine protease (nsp4) are assumed to cleave the ORF1 product into 12 nonstructural proteins (see Fig. 2 and for a review, [45]). Still little is known about the function of the individual nsps. Predictions relating to the functions of the ORF1b-encoded replicase subunits nsp9 and nsp10 are

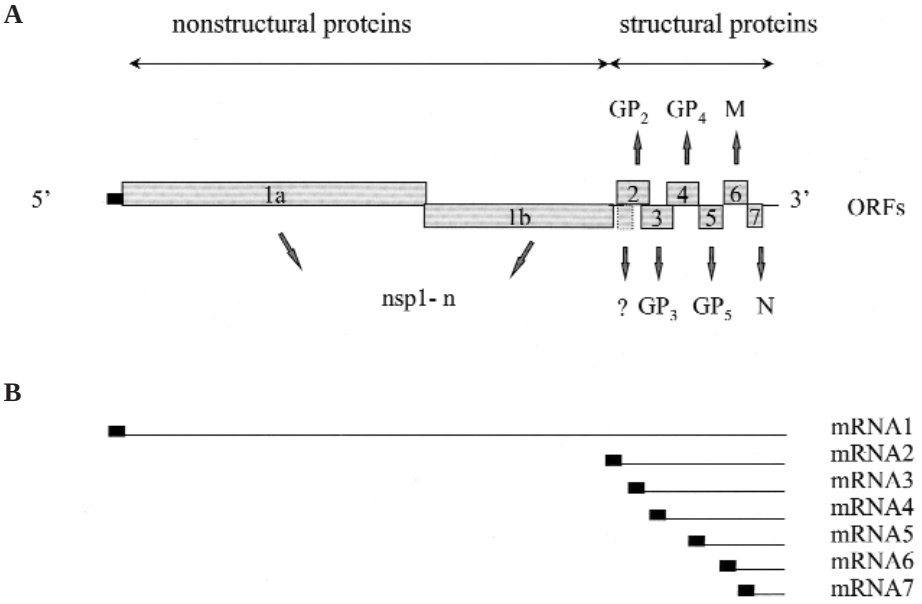

Figure 1. (A) Genome organisation of PRRSV. The replicase gene, consisting of the open reading frames (ORFs) 1a and 1b, encodes a polyprotein that is cleaved, forming smaller protein products designated as nonstructural proteins (nsp1-n). ORF1 is followed by ORFs 2 to 5 encoding glycoproteins GP_2 to GP_5; ORF6 encodes the membrane protein M; and ORF7 encodes the nucleocapsid protein N. An internal ORF present within ORF2 that might encode an additional structural protein is indicated with dashed lines. (B) 3' nested set of subgenomic mRNAs synthesised during PRRSV replication. The 5' leader, derived from the genomic RNA and fused to the subgenomic RNAs, is shown as a black box.

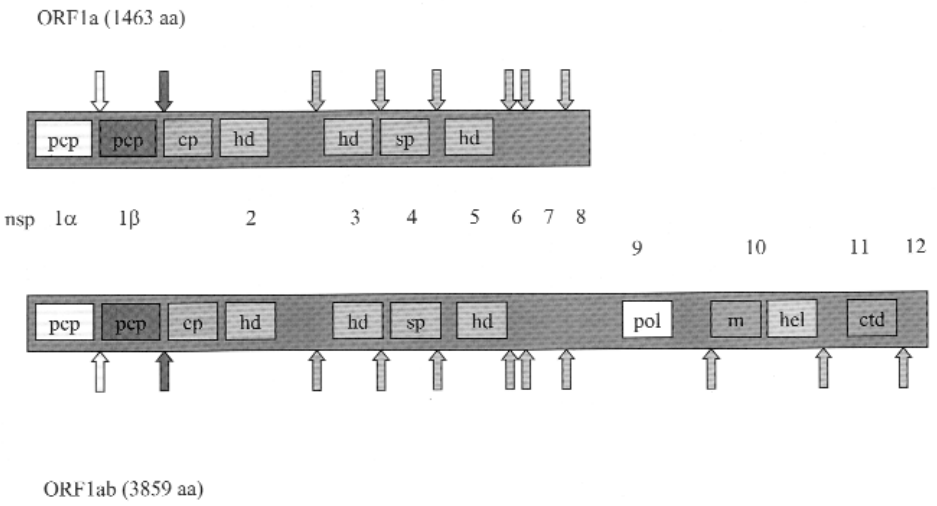

Figure 2. Putative processing scheme of the polyprotein encoded by ORF1a/ORF1ab. The papain-like cysteine proteases (pcp), cysteine protease (cp), and serine protease domains (sp) involved in cleavage of the polyprotein into 12 predicted nonstructural proteins (nsps) are indicated. The putative protease cleavage sites (arrows) are based on sequence comparisons and experimental data obtained for EAV, as reviewed in [45]. Conserved hydrophobic domains (hd), an RNA-dependent RNA polymerase motif (pol), a nucleoside triphosphate-binding/RNA helicase motif (hel), a metal binding domain (m) and conserved 3' terminal domain (ctd) are indicated.

derived from comparative sequence analysis. A putative RNA-dependent RNA polymerase motif was identified in nsp9, and a nucleoside triphosphate-binding/RNA helicase motif and metal binding domain was identified in nsp10 [31].

The six smaller ORFs 2 to 7 located at the 3' end of the genome encode structural proteins that are associated with the virion [32, 33]. The nucleocapsid protein N (encoded by ORF7) and the integral membrane protein M are not N-glycosylated. In contrast, the GP_2, GP_3, GP_4, and GP_5 proteins, which are encoded by ORFs 2, 3, 4, and 5, are all N-glycosylated. Recently, a novel non-glycosylated structural protein, expressed from an ORF located directly downstream of ORF1b and partially overlapping ORF2, was detected in virions of EAV and designated as E [46]. The ORF present within ORF2 of PRRSV might express a homologous protein, since its amino acid sequence has similarity with the E protein of EAV.

ORFs 2 to 7 are expressed from a 3' nested set of subgenomic mRNAs (Fig. 1B). These subgenomic mRNAs are composed of a leader sequence, derived from the 5' end of the viral genome and fused to the subgenomic mRNA bodies by a discontinuous transcription mechanism (reviewed in [45]). The site of fusion, also called the leader-body junction site, is a conserved sequence of six nucleotides: UUAACC (Tab. I).

4. SEQUENCE VARIATION BETWEEN EUROPEAN AND NORTH AMERICAN PRRSV STRAINS

Extensive sequence analysis of field isolates of PRRSV has unexpectedly revealed

Table I. Leader-body junction sequences of different PRRSV strains.

RNA	LV[1]	Distance[2]	VR2332[3]	Distance[2]	ISU79[4]	Distance[2]	EDRD-1[5]	Distance[2]
1	UUAACC	0	UUAACC	0				
2	UAAACC	38	UGAACC	19				
3	UUGACC	11	GUAACC	83	GUAACC	83		
3.1					UUGACC	229		
4.1	UCAACC	83	UUCACC	4	UUCACC	4		
4.2			UCAGCC	56				
5.1	ACAACC	32	UUAGCC	40			UUAGCC	40
5.2			UUAGUC	111				
6	UCAACC	24	AUAACC	17			UUAACC	17
7.1	UUAACC	9	AUAACC	123			AUAACC	123
7.2			UUAACC	9				
con-sensus	UUAACC		UUAACC		UUAACC		UUAACC	

(1) Data derived from [30].
(2) Distance between the leader-body junction sequence and the initiation codon of the downstream ORF.
(3) Data derived from [38].
(4) Data derived from [29].
(5) Data derived from [44].

high sequence variation between North American and European isolates of PRRSV [1, 2, 7, 12, 16, 24, 27, 28, 31, 37, 38, 44, 48]. The GP_5 protein is the most variable structural protein, with only 51-55% amino acid identity between North American and European isolates, whereas the M protein is the most conserved structural protein, with 78-81% amino acid identity (Fig. 3). Sequence comparison between the ORF1 genes of LV and two US strains that were recently sequenced [1, 38] also revealed major differences in this part of the genome. The greatest variation was observed in the amino acid sequence of nsp2. The nsp2 protein of US strains was 102 amino acids longer than that of LV and only shared 32% identical amino acids. Furthermore, differences in the leader-body junction sites that are used by European and US viruses were observed. Although the leader-body junction sequence (UUAACC) is conserved in both European and North American isolates, the distance between the junction sequence and the downstream ORF is highly variable (Tab. I). Furthermore, additional junction sites that are less frequently used were identified for the North American strains VR2332 and ISU96 but not for LV and EDRD-1.

5. STRUCTURAL PROTEINS

PRRSV possesses a large set of five to seven structural proteins, which is rather unusual for positive-strand RNA viruses (Fig. 4). The three major structural proteins GP_5, M, and N are encoded in this order by the three most 3' ORFs of the PRRSV genome [3, 25, 32]. The N protein is small (15 kDa) and highly basic, which may facilitate its interaction with the genomic RNA in the assembly of the nucleocapsids [31]. The regions of N important for this interaction have not yet been defined. The N protein is expressed at high levels in infected cells and constitutes about 20-40% of the protein content of the virion.

The 18-kDa non-glycosylated M protein is the most conserved structural protein of PRRSV. Its structure resembles that of the coronavirus M proteins in that it traverses the membrane three times by means of membrane spanning regions present at the

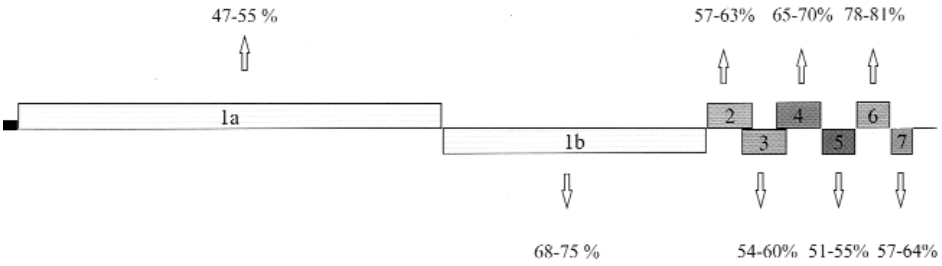

Figure 3. Amino acid sequence identity between the proteins encoded by ORFs 1 to 7 of European and North American strains. Sequence data were derived from [1,2,7,12,16,24,27,28,31,37,38,44,48].

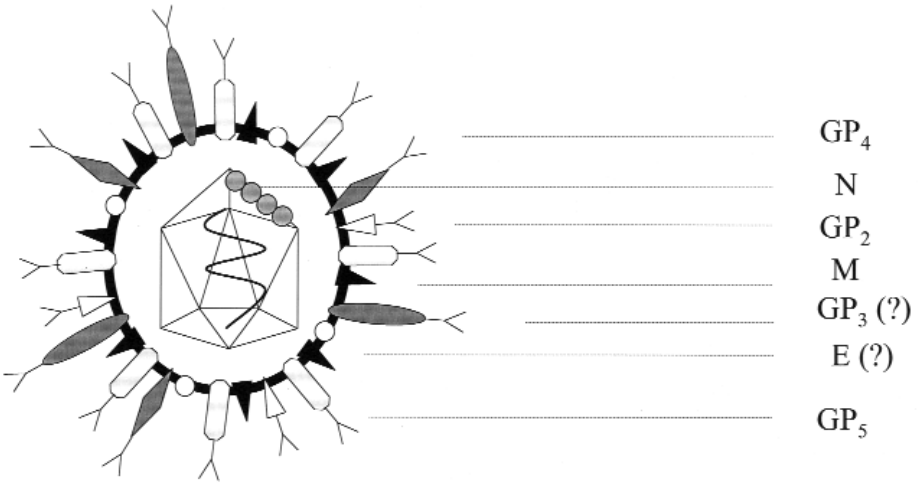

Figure 4. Schematic presentation of the virus particle of PRRSV. The locations of the structural proteins are indicated. The structural nature of the GP_3 protein is questionned, the presence of a putative E protein still needs to be determined.

N terminus. This results in a Nexo-Cendo configuration with a short stretch of only 16 amino acids exposed at the virion surface. One of the membrane spanning fragments is thought to function as a signal sequence. Although little is known about the function of the PRRSV M protein, it might play a role in virus assembly and budding, as has been shown for coronaviruses. The M protein accumulates in the ER, where it forms disulfide-linked heterodimers with the 25-kDa major glycoprotein GP_5 [25]. These heterodimers are incorporated in virus particles and are assumed to be essential for virus infectivity, as has been shown for other arteriviruses [10]. Disulfide-linked homodimers of the M protein have also been detected in infected cells, but these were not incorporated in virions [25].

The GP_5 protein contains an N-terminal signal sequence which is assumed to be cleaved [32]. The processed protein then contains a short putative ectodomain that is approximately 30 amino acids long and

contains two N-glycans in European isolates but three N-glycans in most North American isolates. In contrast to the homologous protein of EAV [10], the GP_5 protein of PRRSV does not contain N-acetyllactosamine repeats [32]. The internal hydrophobic region is predicted to span the membrane three times and is followed by an endodomain of 70 amino acids. Although one might speculate that the primary envelope protein is involved in receptor recognition, no direct experimental evidence is yet available. PRRSV induces in vitro apoptosis in macrophages and in germ cells [47, 49]. The GP_5 protein is thought to play a role in the induction of this process, but the relevance for in vivo pathogenesis is unclear.

The 29-30 kDa GP_2 and the 31-35 kDa GP_4 protein of PRRSV were identified as minor glycoprotein constituents of the virus particle and are typical class I membrane proteins [33, 50]. They contain an N-terminal signal sequence, a C-terminal transmembrane segment and an ectodomain possessing complex-type N-glycans when incorporated into virions. Conflicting data on the presence of the ORF3-encoded glycoprotein GP_3 in particles of PRRSV have been presented. Whereas van Nieuwstadt et al. [50] detected the 45-50 kDa GP_3 protein on Western blots of purified Lelystad virus particles with specific monoclonal antibodies, the GP_3 protein of the Canadian IAF-klop isolate was not detected in the purified virus by immunoprecipitation [26]. The GP_3 protein was however detected in lysates of cells infected with IAF-Klop and appeared to be retained in the ER since its core N-glycans were not converted to complex-type N-glycans. Interestingly, small amounts of the latter protein were detected in a soluble form (sGP_3) in the medium. Unlike GP_3, sGP_3 was folded into disulfide-linked dimers and its N-glycans had acquired Golgi-specific modifications. Further research is needed to explain these contradictory results concerning the nature of the GP_3 protein. In addition it has to be established whether a homologue of the EAV E protein is present in PRRSV virions.

6. ANTIGENICITY OF THE VIRAL PROTEINS

A wide variety of monoclonal antibodies (MAbs) directed against the GP_3, GP_4, GP_5, M, and N proteins have been generated by immunising mice with PRRSV virus preparations [8, 11, 23, 39, 50, 53]. Most MAbs were directed against the N protein suggesting that it is the most immunodominant protein. Alternatively, recombinant proteins of GP_4, GP_5, and N were used to generate MAbs [41, 43, 55]. Different epitopes have been identified on the N protein of PRRSV using these MAbs. Some of the epitopes are specific for European or North American isolates, whereas others are conserved in both subgroups. An immunodominant antigenic domain was mapped in the central region of the N protein: amino acids 50-66 and 80-90 [36, 43, 54]. The binding of MAbs to this domain was disturbed by N-terminal deletions of 11 or 31 amino acids, indicating that the N terminal sequences are important for a proper conformation [36, 54]. MAbs directed to the GP_4 and GP_5 protein neutralised the virus in vitro, suggesting that these proteins may play a role in attachment of the host cell [41, 50, 53]. The MAbs directed against the major envelope protein GP_5 were more effective in virus neutralisation than monoclonal antibodies directed against the minor envelope protein GP_4 [51]. A neutralising MAb-binding domain of the GP_4 protein has been localised in the ectodomain between amino acids 39 and 79 [34]. Since this region is highly heterogeneous among different strains of PRRSV, it is most likely subject to immuno-selection.

Recombinant expression products of the structural proteins have been produced with bacterial expression systems, baculovirus and Semliki forest virus vectors, and other expression systems [4, 14, 15a, 17, 20–22,

25, 33, 34, 36, 41, 43, 50, 53–55]. These recombinant proteins are used to produce polyvalent sera in rabbits and MAbs in mice, as described above. They are also used, in addition to virion preparations and lysates of infected cells, to detect protein-specific antibodies in sera from PRRSV-infected pigs. It was shown that PRRSV-infected pigs contain antibodies directed against the major structural proteins GP_5, M, and N [20, 22, 32]. The antibodies directed to N are generally most abundant. Therefore, this polypeptide appears to be suitable for diagnostic tests. Antibodies directed to GP_2, GP_3, and GP_4 are also detected in sera from PRRSV-infected pigs, but at a more variable level [14, 17, 21, 32–34]. Seroneutralisation correlated with antibodies directed to GP_5 , but not with antibodies directed to GP_3, GP_4, and N [15a]. Studies focussed on the cell mediated immune responses during PRRSV infection showed that T-cell proliferation responses in pigs infected by PRRSV are mainly directed to the M, GP_2 and GP_5 protein [4].

7. INFECTIOUS cDNA CLONE

Recently, an infectious cDNA clone of the LV isolate of PRRSV was generated [35]. When transcripts of this cDNA clone are transfected to BHK-21 cells, progeny virus is produced and secreted into the medium (Fig. 5). The produced virus can be further propagated in porcine alveolar macrophages or CL2621 cells. The transcripts are first transfected to BHK-21 cells because these cells are more efficiently transfected than CL2621 cells or macrophages. Although PRRSV cannot enter BHK-21 cells, once the genomic RNA is introduced into these cells, the virus is produced. Site-specific mutagenesis has been used to introduce restriction sites, amino acid substitutions in antigenic sites, or an antigenic tag in the genome of PRRSV ([15b, 35], Meulenberg unpublished data). This results in mutant PRRSV viruses that can be distinguished from the wild type virus by genetic or immunogenic analysis. However, only a few mutations are tolerated by the virus, indicating that the major part of the

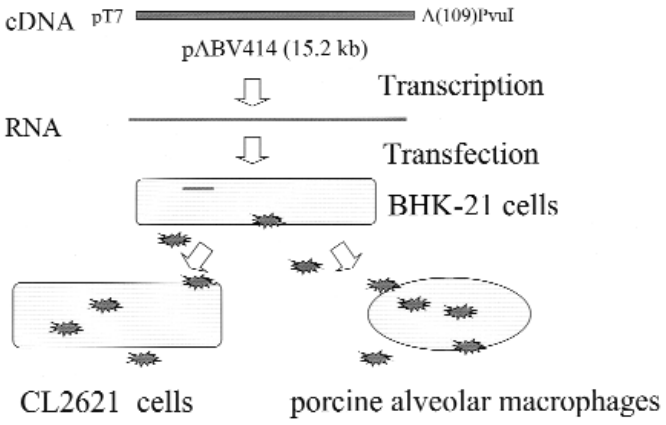

Figure 5. Production of infectious virus from full length cDNA clone pABV414 [35]. The 15.2 kb cDNA clone is linearised with *Pvu*I and transcribed with T7 RNA polymerase. The produced RNA is transfected to BHK-21 cells using lipofectin or electroporation. At 24 h after transfection, the medium of the transfected BHK-21 cells is harvested. The progeny virus is subsequently multiplied in CL2621 cells or porcine alveolar macrophages.

genomic RNA contains essential sequences for virus replication.

8. CONCLUSIONS

Since the discovery of PRRSV in 1991, much has been learned about this virus. The complete genome of different isolates has been sequenced and the availability of cloned PRRSV sequences has greatly increased our understanding of viral RNAs and proteins. The structural proteins associated with the virion have been identified and characterised to a great extent. Several tools, such as an infectious cDNA clone, MAbs, and recombinant PRRSV proteins have been generated. Further studies should focus on the role that individual viral proteins play in the viral replication cycle and in the immunology and pathogenesis of PRRSV infection in the pig.

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