CHAPTER 3.9.6.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME (INFECTION WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS)

SUMMARY

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory problems of piglets and growing pigs. The disease is caused by the PRRS virus (PRRSV), a virus currently classified as a member of the order Nidovirales, suborder Arnidovirineae, family Arteriviridae, subfamily Variarterivirinae, genus Betaarterivirus. The primary target cells of the virus are differentiated macrophages of the pig, mainly pulmonary alveolar macrophages and pulmonary intravascular macrophages, but the virus may also replicate in monocyte-derived macrophages in lymphoid tissues and, to a lesser extent, in dendritic cells and monocyte-derived macrophages residing in most organs in perivascular space. There is an increasing body of evidence that highly pathogenic strains of PRRS virus are also able to infect endothelial cells in lung, heart and brain. This attribute is thought to be associated with higher pathogenicity and may also be of value in characterising new strains.

According to the newest classification, previous genotypes are now considered to be two distinct species named Betaarterivirus suid 1 and Betaarterivirus suid 2, classified within two different subgenera, Eurpobartevirus and Ampobartevirus, respectively. In the present chapter, commonly accepted and recognised conventional names (PRRSV-1 and PRRSV-2) will be used to denote two PRRSV species, respectively. Historically, PRRSV-1 (previously described as genotype 1, Type 1 or European – EU) was restricted to Europe and PRRSV-2 (previously genotype 2, Type 2 or North American – NA) to North America; currently they are distributed globally. The virus is primarily transmitted via direct contact but also by contact with faeces, urine, semen and fomites. The possibility of insect vectors (houseflies and mosquitos) and aerogenic spread for short distances has also been confirmed through this route is considered to be of minor importance. PRRS occurs in most major pig-producing areas throughout the world. The reproductive failure is characterised by infertility, late fetal mummification, abortions, stillbirths, and the birth of weak piglets that often die soon after birth from respiratory disease and secondary infections. Older pigs may demonstrate mild signs of respiratory disease, usually complicated by secondary infections. In 2006, a highly pathogenic PRRSV strain emerged in China (People's Rep. of) causing high fever (40–42°C) in all age groups, abortions in sows and high mortality in sucking piglets, weaners and growers.

Detection of the agent: Virological diagnosis of PRRSV infection is difficult; the virus can be isolated from serum or organ samples such as lungs, tonsils, lymph nodes and spleen of affected pigs. As porcine alveolar macrophages are one of the most susceptible culture systems for virus of both species, these cells are recommended for virus isolation. Recent findings show that porcine monocyte-derived macrophages can also be used for PRRSV isolation and propagation in culture. MARC-145 (MA-104 clone) cells are suitable for isolation of PRRSV-2. There is variability between batches of macrophages in their susceptibility to PRRSV. Thus, it is necessary to identify a batch with high susceptibility, and maintain this stock in liquid nitrogen until required. The virus is identified and characterised by immunostaining with specific antisera or monoclonal antibody. Additional techniques, such as immunohistochemistry and in-situ hybridisation on fixed tissues, reverse-transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR, have been developed for laboratory confirmation of PRRSV infection.

Serological tests: A wide range of serological tests is currently available for the detection of serum, oral fluid and meat juice antibodies to PRRSV. The immunoperoxidase monolayer assay and immunofluorescence assay using alveolar macrophages or MARC-145 cells can be used for the detection of antibodies specific to PRRSV-1 or PRRSV-2. Commercial or in-house enzyme linked immunosorbent assays (ELISA) are now most often used for PRRSV diagnosis. An indirect ELISA and a blocking ELISA have been described as well as a double blocking ELISA, using antigen from both PRRSV-1 and PRRSV-2, that can distinguish between serological reactions to the two species. Commercial ELISAs specifically designed for detection of PRRSV seroconversion in oral fluid also exist.

Requirements for vaccines: Vaccines can be of value as an aid in the prevention or control of reproductive and respiratory forms of PRRS. Vaccination with modified live virus may result in shedding of vaccinal virus in semen and vertical and horizontal transmission between sows and piglets and between vaccinated and non-vaccinated pigs. Subsequent vaccine-virus-induced adverse signs have been reported. Modified live virus vaccines can persist in vaccinated herds. Whole virus inactivated vaccines are also available.

A. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory disease in pigs (as reviewed by Zimmerman *et al.,* 2019). PRRS was first recognised in 1987 in the United States of America, in 1989 in Japan and in 1990 in Germany. Within a few years it became a pandemic. The disease is caused by the PRRS virus (PRRSV), discovered in 1991 in The Netherlands and in 1992 in USA (Zimmerman *et al.,* 2019). PRRSV is a single-stranded positive-sense RNA virus and the biology of the virus has been well characterised. Apart from domestic pigs, feral swine and wild boars, no other species are known to be naturally infected with PRRSV. The virus does not pose a zoonotic risk and it is not infectious for humans or for cells of human origin. Soon after the discovery of the virus it became apparent that the European and North American isolates represent two antigenically distinct groups, initially recognised as two genotypes (Zimmerman *et al.,* 2019). According to the newest, revised classification of the International Committee on Taxonomy of Viruses (ICTV, Virus Taxonomy: 2019 Release), previous genotypes are now considered to constitute two distinct species named Betaarterivirus suid 1 and Betaarterivirus suid 2 and classified within two different subgenuses *Eurpobartevirus* and *Ampobartevirus* in the genus *Betaarterivirus* (subfamily Variarterivirinae, Family Arteriviridae, Suborder Arnidovirinae, Order Nidovirales). In the present chapter, commonly accepted and recognised conventional names (PRRSV-1 and PRRSV-2) will be used to denote two PRRSV species. Additional investigations have demonstrated regional differences within each continent. These differences are now becoming blurred as PRRSV-2 has been introduced into Europe and PRRSV-1 virus has been discovered in North America. Most PRRSV isolates from South America and much of Asia are of species 2 and it is assumed these viruses were introduced through the movement of swine or semen. Most highly virulent PRRSV-2 viruses in South-East Asia (highly pathogenic PRRSV) are characterised by amino acid deletions in the NSP2 region of the genome. However, there is good experimental evidence that these deletions do not determine virulence (Shi *et al.*, 2010a; Zhou *et al.,* 2009; Zhou & Yang, 2010).

There is an increasing diversity among strains of the two species, which has been attributed to the high error rate inherent in PRRSV replication and recombinations between strains (Murtaugh *et al.*, 2010). There have also been descriptions of east European strains of PRRSV-1 with a high degree of polymorphism, providing further insights into the emergence of the relatively new pathogen of pigs. This polymorphism has been proposed to distinguish subtypes 1, 2 and 3 within PRRSV-1. Moreover, mounting evidence indicates that an additional subtype (subtype 4) might exist (Stadejek *et al.,* 2008; 2013). The effects of such diversity on diagnostics and vaccines are largely unknown, but do raise concerns and should be considered. Subtype 3 (Lena, SU1-bel) and subtype 2 Bor strains have been shown to have higher virulence than subtype 1 strains (Karniychuk *et al.*, 2010; Morgan *et al.,* 2013; Stadejek *et al.*, 2017). Trus *et al.* (2014) showed that subtype 1 modified live vaccine partially protects against challenge with subtype 3 Lena strain. Although nine different genetic lineages have been identified in PRRSV-2, the overall level of diversity within species 2 does not exceed that observed for subtype 1 of PRRSV-1 (Shi *et al.*, 2010b; Stadejek *et al.*, 2013).

The reproductive syndrome is recognised by late-gestation abortions and early or delayed farrowings that contain dead and mummified fetuses, stillborn pigs, and weak-born pigs. An increase in repeat breeders during the acute phase of the epizootic is commonly reported. Infrequently, there are reports of early- to mid-gestation reproductive failure. Most probably the cause of PRRSV-related reproductive disorders is virus-induced damage to the placenta and endometrium (Karniychuk & Nauwynck, 2013). In boars and unbred replacement gilts and sows, transient fever

and anorexia may be observed. The respiratory syndrome is recognised by dyspnoea ("thumping"), fever, anorexia, and listlessness. Younger pigs are more affected than older animals with boars and sows (unbred) frequently having subclinical infection. An increase in secondary infections is common and mortality can be high. In PRRSV-infected boars and boars that have been vaccinated with live attenuated vaccine, PRRSV can be shed in semen, and changes in sperm morphology and function have been described (Christopher-Hennings *et al.,* 1997). The virus is primarily transmitted directly via contact with infected pigs but also with faeces, urine and semen. It can also be spread by insects (houseflies and mosquitoes) and indirectly, presumably via aerosol routes, leading to chronic re-infections of herds in swine dense areas, and possibly by mechanical vectors. Gross and microscopic lesions consistent with PRRSV infection have been well described (Zimmerman *et al.,* 2019). In general, the lesions are more severe in younger animals than older ones. Differences in virulence between PRRSV isolates within a genotype and between genotypes were proved to exist based on field observations and experimental studies (Karniychuk *et al.*, 2010; Stadejek *et al.* 2017; Weesendorp *et al.*, 2013). This variability has been reinforced with the emergence in 2006 of a PRRSV lineage in South-East Asia associated with porcine high fever disease, a syndrome causing high mortality in all ages of swine (Tian *et al.*, 2007). Although there is now an extensive body of research completed since the discovery of PRRSV, there are still many gaps in the knowledge base about the apparent link between PRRSV and other diseases as well as understanding the PRRSV immune response.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of porcine reproductive and respiratory syndrome and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Virus isolation	–	++	–	+++	–	–
Conventional RT-PCR	+++	+++	+++	+++	++	–
Real-time RT-PCR	+++	+++	+++	+++	++	–
IHC	–	–	–	++	–	–
ISH	–	–	–	++	–	–
Detection of immune response[b]						
ELISA	+++	++	+++	++	+++	++
IPMA	++	++	++	+	++	+++
IFA	++	++	++	+	++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; IHC = immunohistochemistry method;
ISH = *in-situ* hybridisation; ELISA = enzyme-linked immunosorbent assay; IPMA = immunoperoxidase monolayer assay,
IFA = immunofluorescence assay.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[b]One of the listed serological tests is sufficient.

1. Detection of the agent

Identification of PRRSV can be accomplished by virus isolation, the detection of nucleic acids, and the detection of viral proteins. Following infection, swine develop a viraemia and lung infection that can persist for weeks in young pigs and days in adult animals making serum and bronchoalveolar lung lavage ideal samples to collect for detection of PRRSV.

1.1. Virus isolation

Isolation of PRRSV can be difficult as not all virus isolates (especially PRRSV-1 viruses) can easily infect MARC-145 cells and CL-2621, clones derived from the MA-104 monkey kidney cell line (Provost *et al.*, 2012; Zimmerman *et al.*, 2019). Recent findings show that porcine monocyte-derived macrophages can also be used for PRRSV isolation and propagation in cell culture (Garcia-Nicolas *et al.*, 2014). These can be differentiated *in vitro* from porcine peripheral blood mononucleated cells (PBMCs) without slaughtering animals, as opposed to collection of the lung for porcine alveolar macrophage (PAM) preparations. Moreover, several genetically modified cell lines supporting PRRSV replication have been developed including immortalised PAM cell line expressing CD163, immortalised porcine monomyeloid cells, PK-15 expressing CD163 and sialoadhesin as well as porcine, feline and baby hamster kidney cells expressing CD163 (Delrue *et al.*, 2010; Provost *et al.*, 2012). Other, non-recombinant cell lines permissive for PRRSV infection have also been described (Feng *et al.*, 2013; Provost *et al.*, 2012). PAM will support replication of most, if not all PRRSV isolates. However, the collection of PAM is not an easy task as only pigs of high health status and less than 8 weeks of age should be used as the PAM source (Feng *et al.*, 2013). Different batches of PAM are not always equally susceptible to PRRSV; it is thus necessary to test each batch before use. PAM can be stored in liquid nitrogen until needed as described below. Isolation of PRRSV using PAM is a technique that can be performed in most diagnostic laboratories. This technique should be sensitive for isolation of all PRRSV strains and will be explained in detail. Samples for virus isolation should be refrigerated at 4°C immediately after collection and shipped to the laboratory within 24–48 hours. The half-life of the virus in serum at this temperature was estimated as 155 hours. However, infectivity is rapidly lost outside of pH 6.5–7.5 range (Zimmerman *et al.*, 2019). For longer storage freezing at –70°C is recommended.

1.1.1. Harvesting of alveolar macrophages from lungs

Lungs should preferably be obtained from specific pathogen free pigs or from a herd of pigs that is proven to be free from PRRSV infection. Best results are obtained with pigs that are under 8 weeks of age. The macrophages should be harvested from the lung on the same day that the pig is slaughtered. The lungs should be washed three or four times with a total volume of approximately 200 ml sterile phosphate-buffered saline (PBS). The harvested wash fluid is then centrifuged for 10 minutes at 300 g. The resulting pellet of macrophages is resuspended in PBS and centrifuged (washed) twice more. The final pellet is resuspended in 50 ml PBS, and the number of macrophages is counted to determine the cell concentration. The macrophages can then be used fresh, or can be stored in liquid nitrogen according to standard procedures at a final concentration of approximately 6×10^7 macrophages/1.5 ml vial. Macrophage batches should not be mixed.

1.1.2 Batch testing of alveolar macrophages

Before a batch of macrophages can be used it should be validated. This should be done by titrating a standard PRRSV with known titre in the new macrophages, and by performing an immunoperoxidase monolayer assay (IPMA) with known positive and negative sera on plates seeded with the new macrophages. The cells are suitable for use only if the standard PRRSV grows to its specified titre, (TCID$_{50}$ or 50% tissue culture infective dose). Alveolar macrophages and fetal bovine serum (FBS) to supplement culture medium must be pestivirus free.

1.1.3 Virus isolation/titration on alveolar macrophages

Alveolar macrophages are seeded in the wells of flat-bottomed tissue-culture grade microtitre plates. After attachment, the macrophages are infected with the sample. Samples can be sera or 10% suspensions of tissues, such as tonsils, lung, lymph nodes, and spleen. In general, the PRRSV gives a cytopathic effect (CPE) in macrophages after 1–2 days of culture, but sometimes viruses are found that give little CPE or give a CPE only after repeat passage. After a period of 1–2 days or

once CPE has been observed, the presence of PRRSV needs to be confirmed by immunostaining with a specific antiserum or monoclonal antibody (MAb).

i) Seeding macrophages in the microtitre plates

Defrost one vial containing 6×10^7 macrophages/1.5 ml. Wash the cells once with 50 ml PBS and centrifuge the cell suspension for 10 minutes at 300 g (room temperature). Collect the cells in 40 ml RPMI 1640 medium supplemented with 1% glutamine, 10% FBS and 1–2% antibiotic mixture (growth medium). Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of 10^5 cells in each well of the plates).

ii) Preparation of sample (serum, 10% tissue suspension) dilutions in a dummy plate

Dispense 90 µl of growth medium into each well of a microtitre plate. Add 10 µl samples to the wells of rows A and E (duplicate 1/10 dilution). Shake the plates and transfer 10 µl from rows A and E to rows B and F (1/100 dilution). Shake the plates and transfer 10 µl from rows B and F to rows C and G (1/1000 dilution). Shake the plates and transfer 10 µl from rows C and G to rows D and H (1/10,000 dilution). Shake the plates. For virus isolation without titration, dilutions of 1/10 and 1/100 are sufficient.

iii) Incubation of samples

Transfer 50 µl of the sample dilutions from the dilution plates to the corresponding wells of the plate with macrophages (first passage). Incubate for 2–5 days and observe daily for a CPE. At day 2, seed macrophages in new microtitre plates (see above). Transfer 25 µl of the supernatants from the plates of the first passage to the corresponding wells of the freshly seeded plates (second passage). Incubate for 2–5 days and observe daily for a CPE.

iv) Reading and interpreting the results

Wells in which macrophages show CPE in the first passage only are considered to be false positive because of the toxicity of the sample. Wells in which macrophages show CPE in both passages, or in the second passage only, are considered to be suspect positive. All wells with macrophage monolayers that do not show CPE need to be identified as PRRSV negative by immunostaining with a PRRSV-positive antiserum or MAb. CPE-positive samples need to be identified as PRRSV positive by culturing CPE-positive supernatant samples, or the original sample dilutions, for both 24 and 48 hours in macrophages, followed by immunostaining with a PRRSV-positive antiserum or MAb.

v) Immunostaining with a PRRSV-positive antiserum or MAb

Infect macrophages with 50 µl of supernatant or tissue sample as described in Section B.2.1, and grow the infected cells for 24 and 48 hours. Prepare an appropriate dilution of a PRRSV-positive serum in dilution buffer, and immunostain the macrophages as described in Section B.2.1 or B.2.2.

1.2. RNA detection methods

One of the most commonly used diagnostic techniques is detection of PRRSV nucleic acid with reverse-transcription polymerase chain reaction (RT-PCR), nested RT-PCR, and real-time RT-PCR (Kleiboeker *et al.,* 2005; Wernike *et al.*, 2012a; 2012b). The advantages of RT-PCR are high specificity and sensitivity as well as rapid evaluation of a current infection status. However, inactivated virus cannot be differentiated from infectious virus using this technique. RT-PCR-based tests are commonly used to detect nucleic acid in tissues and serum. It has been suggested that oral fluids testing also give reliable results for pen-based diagnosis (Kittawornrat *et al.*, 2010). Another population-based sample that can be used for monitoring PRRSV infections in breeding herds is processing fluid, obtained as an exudate from tissues collected during piglet castration and tail-docking (Zimmerman *et al.*, 2019). The above-mentioned assays are also useful when virus isolation is problematic, such as when testing semen (Christopher-Hennings *et al.,* 1997) and when testing tissues partially degraded by autolysis or by heat during transport of specimens for virus isolation. Most of the in-house protocols and currently available commercial kits provide the possibility of differentiating species of PRRSV-1 and 2 (Kleiboeker *et al.*, 2005; Wernike *et al.*, 2012a;

2012b). False-negative results related to high genetic diversity, and primer and probe mismatches are the major concern when using RT-PCR. Currently, no single RT-PCR assay is capable of detecting all PRRSV strains, especially within highly diverse European subtypes 2–4 of PRRSV-1. RT-PCR results should be carefully evaluated, and continual validation based on recently circulating PRRSV strains is strongly recommended (Wernike *et al.*, 2012a). Reverse-transcription – loop-mediated isothermal amplification (RT-LAMP) is an alternative technique not requiring advanced equipment unlike the real-time RT-PCR (Zimmerman *et al.*, 2019). All of these nucleic acid tests are more rapid than virus isolation and do not require cell culture infrastructure, with the added advantage of differentiating between species.

The following example RT-PCR protocols allow for differentiation between PRRSV-1 and PRRSV-2 based on the gel electrophoresis of amplified fragments (conventional RT-PCR) or specific fluorescent-labelled probes (real-time RT-PCR). The oligonucleotides presented were shown to recognise a wide range of diverse PRRSV strains (Wernike *et al.*, 2012a). However, when relying on RT-PCR methods it is important to determine the sequence of locally circulating strains, and, if necessary, to adjust the primers and probe sequences accordingly. Cycling conditions should be adapted depending on the exact composition of the reaction mix, enzymes characteristics and thermal cycler applied. Positive (PRRSV-1 and PRRSV-2) and negative controls should be included in every run. Additional controls – positive and negative extraction controls and internal controls for real-time PCR – are recommended. To minimise the possibility of contamination, separation of consecutive protocol stages (RNA extraction, assembling the reaction mix, adding the RNA template, gel electrophoresis) is highly recommended.

1.2.1. Conventional RT-PCR

i)   Perform RNA extraction.

ii)   Reverse transcription:

Reverse transcription may be performed separately (e.g. 30 minutes at 50°C), or included as a part of a one-tube RT-PCR reaction, as indicated below.

iii)   Prepare the reaction mix for a one-step RT-PCR (final volume 25 µl)

RT-PCR buffer	1× concentrated (including 2.5 mM $MgCl_2$)
dNTPs mix	final concentration 0.4 mM
Forward and reverse primers	final concentration of each primer 0.5 µM

Primers amplify partial ORF7/3'UTR region of PRRSV (Wernike *et al.*, 2012a):

Forward primer	5'-ATG-GCC-AGC-CAG-TCA-ATCA-3'
Reverse primer	5'-TCG-CCC-TAA-TTG-AAT-AGG-TGA-CT-3'
RT-PCR enzyme mix	Recommended No. of Units of each enzyme
RNA	5 µl
RNase-free water	up to 25 µl

iv)   Perform a one-step RT-PCR reaction according to the following thermal protocol:

Reverse transcription 50°C for 30 minutes

Reverse transcriptase inactivation and polymerase activation: depending on the enzyme characteristics

35 cycles of

Denaturation	94°C for 30 seconds
Annealing	55°C for 40 seconds
Extension	72°C for 50 seconds

Final extension   72°C for 10 minutes

v)   Gel electrophoresis

Analyse obtained amplicons through electrophoresis on a 1.5% agarose, staining with an intercalating dye (ethidium bromide or its equivalent) and visualisation in a UV light.

Differentiation between PRRSV-1 and PRRSV-2 is based on the length of the amplicons, which are 398 bp and 433 bp, respectively

1.2.2. Real-time RT-PCR

i) Perform RNA extraction

ii) Reverse transcription

Reverse transcription stage may be performed separately (e.g. 30 minutes at 50°C) or included as a part of a one-tube RT-PCR reaction.

iii) Prepare the reaction mix using the reagents appropriate for hydrolysis probe-based detection. Most often, the reaction is performed in a volume of 25 µl, including 2-5 µl of extracted RNA. The final concentration of primers and probes should be approximately 0.4 µM and 0.2 µM, respectively.

Primers and probes sequences (Wernike *et al.,* 2012a)

PRRSV-1 (ORF6, ORF7)

EU-1 Forward primer 5'-GCA-CCA-CCT-CAC-CCR-RAC-3'

EU-2 Forward primer 5'-CAG-ATG-CAG-AYT-GTG-TTG-CCT-3'

EU-1 Reverse primer 5'-CAG-TTC-CTG-CRC-CYT-GAT-3'

EU-2 Reverse primer 5'-TGG-AGD-CCT-GCA-GCA-CTT-TC-3'

Probe EU-1 5'-(6-FAM)-CCT-CTG-YYT-GCA-ATC-GAT-CCA-GAC-(BHQ1)-

Probe EU-2 6-FAM-ATA-CAT-TCT-GGC-CCC-TGC-CCA-YCA-CGT-BHQ1

PRRSV-2 (ORF7, 3'UTR)

NA Forward primer 5'-ATR-ATG-RGC-TGG-CAT-TC-3'

NA Reverse primer 5'-ACA-CGG-TCG-CCC-TAA-TTG-3'

NA Probe 5'-(TEX)-TGT-GGT-GAA-TGG-CAC-TGA-TTG-ACA-(BHQ2)-3'

iv) Example thermal protocol for one-step real-time RT-PCR reaction

Reverse transcription   50°C for 30 minutes

Reverse transcriptase inactivation/polymerase activation: 95°C for 15 minutes

45 cycles of

Denaturation	94°C for 15 seconds
Annealing	60°C for 60 seconds
Extension	72°C for 10 seconds

Final extension   72°C for 10 minutes

v) Interpretation of results

The results may be considered positive if:

a) Fluorescence curves show a typical sigmoidal shape,

b) Fluorescence in the channels dedicated to PRRSV-1 and PRRSV-2 exceeds the level of background fluorescence indicated by the threshold line (note that unspecific fluorescence may occur in several last cycles due to the probes degradation). The number of cycles required for the fluorescent signal to cross the threshold line is expressed as a Ct value.

c) Positive controls give a positive signal in the designated channel and no increase of fluorescence in negative controls are observed.

If the above-mentioned conditions are not met, the test should be repeated.

1.3. Other methods

Although seldom used for diagnostic purposes, *in-situ* hybridisation is capable of detecting and differentiating PRRSV-1 and -2 in formalin-fixed tissues. The sensitivity and specificity of this method for detection of PRRSV genome can be compromised by the very high genetic diversity of PRRSV, especially within PRRSV-1. Immunohistochemistry can be used to identify viral proteins and when performed on formalin-fixed tissues enables the visualisation of antigen together with histological lesions (Zimmerman *et al.,* 2019).

Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified products was developed and used for the differentiation of field and vaccine PRRSV isolates (Zimmerman *et al.,* 2019). However, this method has proven to be of limited value in characterising genetic relationships between strains and their pathogenicity. Although RFLP remains in use, for example for identifying epidemiological links among herds in close proximity, it has been increasingly replaced by sequencing, which allows the study of molecular epidemiology and genetic relatedness between PRRSV strains. Sequencing can also contribute to intervention strategies as a tool for monitoring herd level viral changes over time and differentiation of wild-type and vaccine strains (Zimmerman *et al.,* 2019).

The most common target used for sequencing is the highly variable ORF5 encoding the major envelope glycoprotein (GP5) associated with virus neutralisation. However, with 603/606 nt, it only covers 4% of the viral genome and 12% of structural genes. No correlation was demonstrated between phylogenetic grouping based on ORF5 sequences and pathogenicity, or cross-protection. Therefore this approach cannot be used for prediction of strain virulence or selection of the most efficacious vaccine. Additionally, the high rate of recombination events observed in the field may influence the results of phylogenetic analysis based on such short genome fragments and lead to misclassification of recombinant strains (Martin-Valls *et al.*, 2014).

Despite those concerns, ORF5 sequencing is widely used to gain insight into the genetic structure of PRRSV strains via comparison to the large library of ORF5 sequences deposited in public databases. Most of the reported PRRSV phylogenetic studies are based on data from the ORF5 region, but comparative analyses proved that whole genome sequencing allows for better understanding of viral evolution, improved epidemiological monitoring and classification of strains (Lalonde *et al.*, 2020; Martin-Valls *et al.*, 2014). Currently limited data on PRRSV full-length genome sequences are available, but the rapid development of high throughput sequencing technology stimulates a dynamic progress in this field.

131. **ORF5 sequencing PCR**

    i)   Primers:

         Multiple primer sequences suitable for ORF5 sequencing are available in the literature (Wernike *et al.*, 2012a). Due to the high genetic diversity of PRRSV and accumulation of point mutations, some primers may not be optimal for certain strains; when false negative results are obtained from PCR sequencing, an alternative set of primers should be applied.

         The following protocol is based on a set of primers used by Balka *et al.* (2018):

         PRRSV-1:   Forward primer   5'-TTC-ACA-GAT-TAT-GTG-GCC-CAT-GTG-AC-3'

                    (alternatively   5'-GCG-TYA-CRG-ATT-ATG-TGG-CYC-AYG-T-3')

                    Reverse primer   5'-CGY-GAC-ACC-TTR-AGG-GCR-TAT-ATC-AT-3'

         PRRSV-2:   Forward primer   5'-GTC-AAY-TTT-ACC-AGY-TAY-GTC-CAA-CA-3'

                    Reverse primer   5'-AGR-GCA-TAT-ATC-ATY-ACY-GGC-GTG-TA-3'

    ii) Isolate RNA from body fluids or tissue samples with high to moderate amounts of PRRSV.

    iii) Prepare the reaction mix using an appropriate buffer and one-step RT-PCR enzyme mix (alternatively perform separate RT and PCR reactions). Use 10 pmol of forward and reverse primer per reaction. If not premixed in the buffer, add dNTPs to the final concentration of 400 µM. Optionally add RNAse inhibitor in recommended concentration. Supplement the reaction mix with RNase-free water up to 25 µl volume per reaction.

iv)   Aliquot 22 µl of the prepared reaction mix to the PCR tubes, add 3 µl of sample RNA. Add positive and negative reaction controls to separate tubes.

v)   The example protocol for one-step RT-PCR reaction includes the following steps: 50°C for 30 minutes; 95°C for 15 minutes; 35× (94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute), 72°C for 10 minutes (the temperatures may need to be adjusted according to the enzymes and buffers used).

vi)   Check if the obtained product has expected size by comparing to positive control and marker bands after an electrophoresis on a 1.5% agarose gel.

vii)   Submit the PCR product for further purification, sequencing and phylogenetic analysis.

2.   Serological tests

A variety of assays for the detection of serum antibodies to PRRSV has been described. Serological diagnosis is, in general, easy to perform, with good specificity and sensitivity on a herd basis. Sera collected at a single time point may have limited diagnostic value as results can be influenced by maternal antibodies or previous vaccination. To diagnose active infection in an individual animal, acute and convalescent serum samples can be tested to demonstrate seroconversion. Serology is generally performed with a binding assay, such as the immunoperoxidase monolayer assay (IPMA), immunofluorescence assay (IFA), or the enzyme-linked immunosorbent assay (ELISA) – of which many varieties are described (Diaz *et al*., 2012; Jusa *et al.,* 1996; Sorensen *et al.,* 1998; Venteo *et al*., 2012; Yoon *et al.,* 1992). These tests are often performed with viral antigen of one species, which means that antibodies directed against the other, heterologous species may be detected with less sensitivity. A blocking ELISA has been used extensively in Denmark and has been described as a double ELISA set-up using both PRRSV-1 and -2 viruses as antigens and thus it can distinguish between serological reaction to both species (Sorensen *et al.,* 1998). This is of high importance as PRRSV-2 strains circulate in Europe following PRRSV-2-based modified live vaccine use and independent introduction (Stadejek *et al.,* 2013). The identification of PRRSV-1 strains in North America and Asia has also been reported (Kleiboeker *et al*., 2005; Zimmerman *et al.,* 2019). The prevalence of PRRSV-2 infections in Europe and PRRSV-1 infections in North America and Asia is not well documented. As both types of PRRSV are globally spread, serological tests should contain antigens from both species. Commercial ELISAs with good sensitivity and specificity are available and have been compared (Biernacka *et al.,* 2018; Diaz *et al*., 2012, Venteo *et al*., 2012).

Antibodies to the virus can be detected by antibody-binding assays as early as 7–14 days after infection, and antibody levels reach maximal titres by 30–50 days. Some pigs may become seronegative within 3–6 months, but others remain seropositive for much longer. Antibodies to PRRSV have also been detected in muscle transudate and oral fluid. Neutralising antibodies develop slowly and do not reach high titres. They can appear from 3 to 4 weeks after infection and persist for 1 year or more, or remain undetected. The use of complement to increase the sensitivity of the serum virus neutralisation test has been reported (Jusa *et al*., 1996). Extensive research into the duration of antibody titres after infection has not yet been done, and the results probably depend on the test used. Maternal antibodies have a half-life of 12–14 days, and maternal antibody titre can, in general, be detected until 4–8 weeks after birth, depending on the antibody titre of the sow at birth and the test used. In an infected environment, pigs born from seropositive females can seroconvert actively from the age of 3–6 weeks.

This chapter describes the IPMA in detail as this test can easily be performed in laboratories where virus isolation procedures using macrophages have been established, and can be used with virus of both species. This assay can also be adapted to the MARC-145 cell line for both species (Jusa *et al.,* 1996). An indirect immunofluorescence assay (IFA) using MARC-145 cells can also be performed for PRRSV serology and is included in the present chapter.

2.1.   Detection of antibodies with the immunoperoxidase monolayer assay

Alveolar macrophages are seeded in the wells of microtitre plates. After attachment, the macrophages are infected with PRRSV. The objective is to infect approximately 30–50% of the macrophages in a well so as to be able to distinguish nonspecific sera. After an incubation period, the macrophages are fixed and used as a cell substrate for serology. An alternative method is to use MARC 145 cells instead of macrophage cells. On each plate, 11 sera can be tested in duplicates. Tested sera are diluted and incubated on the cell substrate. If antibodies are present in the tested serum, they will bind to the antigen in the cytoplasm of the macrophages. In the next incubation step, the bound antibodies will be detected

by an anti-species horseradish-peroxidase (HRPO) conjugate. Finally, the cell substrate is incubated with a chromogen/substrate[1] solution. Reading of the test is done with an inverted microscope.

2.1.1. Seeding macrophages in the microtitre plates

i) Defrost one vial containing 6×10^7 macrophages/1.5 ml.

ii) Wash the cells once with 50 ml of PBS and centrifuge the cell suspension for 10 minutes at 300 g (room temperature).

iii) Collect the cells in 40 ml RPMI 1640 medium supplemented with 1% glutamine, 10% FBS, 100 IU (International Units) penicillin and 100 µg streptomycin per ml (growth medium).

iv) Dispense 100 µl of the cell suspension into each well of a microtitre plate (with one vial of cells, four plates can be seeded at a concentration of 10^5 cells in each well of the plates).

v) Incubate the plates for 18–24 hours at 37°C in a 5% CO_2 incubator, under humid conditions. Alternatively, use HEPES buffer (N-2-hydroxyethylpiperazine, N-2-ethane-sulphonic acid) in the medium.

2.1.2. Infection of cells with PRRSV

i) Add to each well 50 µl of a virus suspension containing 10^5 $TCID_{50}$/ml, but leave two wells uninfected to act as controls.

ii) Incubate the plates for 18–24 hours at 37°C in a 5% CO_2 incubator.

2.1.3. Fixation of the cells

i) Discard the growth medium and rinse the plates once in saline.

ii) Knock the plates gently on a towel to remove excess liquid and then dry them (without lid) for 45 minutes at 37°C.

iii) Freeze the plates (without a lid) for 45 minutes at –20°C. (Plates that are not used immediately for testing must be sealed and stored at –20°C.)

iv) Incubate the cells for 10 minutes at room temperature with cold 4% paraformaldehyde (in PBS). Alternatively the cells could be fixed in ice-cold absolute ethanol for 45 minutes at 5°C or in ice-cold 80% acetone for 45 minutes.

v) Discard the paraformaldehyde and rinse the plates once in saline.

2.1.4. Preparation of serum dilutions in a dilution plate

i) Dispense 180 µl of 0.5 M NaCl with 4% horse serum and 0.5% Tween 80, pH 7.2 (dilution buffer), to the wells of rows A and E of the dummy plate(s).

ii) Dispense 120 µl of dilution buffer to all other wells.

iii) Add 20 µl of the test serum or control sera to the wells of rows A and E (= 1/10 dilution), and shake.

iv) Dilute the sera four-fold by transferring 40 µl from rows A and E to rows B and F, and so on to provide further dilutions of 1/40, 1/160 and 1/640.

---

1   *Preparation of chromogen solution*
Stock solution of chromogen (3-amino-9-ethyl-carbazole [AEC]): (a) 4 mg AEC; (b) 1 ml N,N-dimethyl-formamide.
Dissolve (a) in (b) and store the AEC stock solution at 4°C in the dark.
Preparation of chromogen/substrate solution (*prepare shortly before use*)
Prepare 0.05 M sodium acetate buffer, pH 5.0, as follows: Dissolve 4.1 g sodium acetate in 1 litre distilled water. Adjust the pH to 5.00 with 100% acetic acid. Add 1 ml AEC stock solution to 19 ml of 0.05 M sodium acetate buffer. Add 10 µl 30% H_2O_2 for each 20 ml of chromogen/substrate solution. Filter the solution through a 5 µm filter.

2.1.5. Incubation of sera in the plate with fixed macrophages

i) Transfer 50 μl from each of the wells of the dummy plate(s) to the corresponding wells of the plate with the fixed macrophages. Seal the plate(s) and incubate for 1 hour at 37°C.

ii) Discard the serum dilutions and rinse the plate(s) three times in 0.15 M NaCl + 0.5% Tween 80.

2.1.6. Incubation with conjugate

i) Dilute the rabbit-anti-swine (or anti-mouse, if staining isolation plate with MAb) HRPO conjugate to a predetermined dilution in 0.15 M NaCl + 0.5% Tween 80. Add 50 μl of the conjugate dilution to all wells of the plate(s). Seal the plate(s) and incubate for 1 hour at 37°C. Rinse the plates three times.

2.1.7. Staining procedure

i) Dispense 50 μl of the filtered chromogen/substrate (AEC) solution to all wells of the plate(s) (see footnote 3).

ii) Incubate the AEC for at least 30 minutes at room temperature.

iii) Replace the AEC with 50 μl of 0.05 M sodium acetate, pH 5.0 (see footnote 3).

2.1.8. Reading and interpreting the results

If antibodies are present in the test serum, the cytoplasm of approximately 30–50% of the cells in a well are stained deeply red by the chromogen. A negative test serum is recognised by cytoplasm that remains unstained. A serum that reacts nonspecifically might stain all cells in a well (compared with a positive control serum). The titre of a serum is expressed as the reciprocal of the highest dilution that stains 50% or more of the wells. A serum with a titre of <10 is considered to be negative. A serum with a titre of 10 or 40 is considered to be a weak positive. Often nonspecific staining is detected in these dilutions. A serum with a titre of ≥160 is considered to be positive.

2.2. Detection of antibodies with the indirect immunofluorescence assay

Although there is no single standard accepted immunofluorescence assay in use at this time, several protocols have been developed and are used by different laboratories in North America. The IFA can be performed in microtitre plates or eight-chamber slides using the MARC-145 cell line and a MARC-145 cell-line-adapted PRRSV isolate. To prevent cross-reactivity with pestivirus, it is recommended that cells and FBS, to supplement culture medium, be pestivirus free. After an incubation period, PRRSV-infected cells are fixed and used as a cell substrate for serology. Serum samples are tested at a single screening dilution of 1/20 and samples are reported as being negative or positive at this dilution. Each porcine serum to be tested is added to wells or chambers containing PRRSV-infected cells. Antibodies to PRRSV, if present in the serum, will bind to antigens in the cytoplasm of infected cells. Following this step, an anti-porcine-IgG conjugated to fluorescein is added, which will bind to the porcine antibodies that have bound to PRRSV antigens in the infected cells. The results are read using a fluorescence microscope. Microtitre plates may also be prepared for serum titration purposes (see Section B.2.3 below).

2.2.1. Seeding and infection of MARC-145 cells in microtitre plates

i) Add 50 μl of cell culture medium (e.g. Minimal Essential Medium [MEM] containing 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100/ml μg streptomycin) without FBS to each well of columns 2, 4, 6, 8, 10 and 12 of a 96-well plate using a multichannel pipettor.

ii) Trypsinise confluent MARC-145 cells (grown in culture flasks) to be used for seeding 96-well microtitre plates and resuspend cells in cell culture medium containing 8% FBS at a concentration of 100,000–125,000 cells/ml. The MARC-145 cells are trypsinised from culture flasks for IFA once a week using trypsin/EDTA (ethylene diamine tetra-acetic acid) and are seeded in culture flasks at a concentration of 250,000 cells/ml. After 4 days in culture flasks, new cell culture medium containing 2% FBS is added for 3 additional days.

iii) Using a multichannel pipettor, add 150 µl of the cell suspension to each well of the 96-well plate.

iv) Dilute PRRSV preparation in MEM without FBS to $10^{2.2}$ TCID$_{50}$/50 µl and distribute 50 µl in each well of columns 1, 3, 5, 7, 9 and 11.

v) Incubate the plates for approximately 48–72 hours at 37°C in a humidified 5% CO$_2$ incubator to obtain a monolayer with approximately 40–50% of the cells infected as determined by indirect immuno-fluorescence. Alternatively, microtitre plates may first be seeded with MARC-145 cell suspensions (e.g. concentration of 100,000 cells/ml in medium supplemented with 5–10% FBS) and incubated for up to 72 hours until they are confluent. Then volumes of 50 µl of PRRSV preparations (e.g. 10^5 TCID$_{50}$/ml) are added per well and the plates are incubated for an additional 48–72 hours prior to fixation. The use of organic buffers such as HEPES in medium has been suggested to stabilise the pH when CO$_2$ incubators are not available.

2.2.2. Seeding and infection of MARC-145 cells in eight-chamber glass slides

i) Add 500 µl of a MARC-145 cell suspension (e.g. in MEM supplemented with 10% FBS) at a concentration of 100,000 cells/ml to each chamber of eight-chamber glass slides.

ii) Incubate the cells for approximately 48–72 hours at 37°C in a humidified 5% CO$_2$ incubator until they are confluent.

iii) Add to each chamber 50 µl of PRRSV suspension containing 10^5 TCID$_{50}$/ml and further incubate cells for approximately 18 hours at 37°C in a humidified 5% CO$_2$ incubator . At this time 15–20 infected cells per field of view may be observed by indirect immunofluorescence.

2.2.3. Fixation of the cells

i) Discard the medium, rinse once with PBS and discard the PBS. For chamber slides, remove the plastic chamber walls, leaving the gasket intact.

ii) Add volumes of 150 µl cold (4°C) acetone (80% in water) to each well of the 96-well plate. Incubate the plates at 4°C for 30 minutes. For chamber slides, acetone (80–100%) at room temperature is used to fix the cells for 10–15 minutes at room temperature. Some manufactured brands of acetone will degrade the chamber slide gasket leaving a film on the slide. It is recommended to check the acetone before using for routine fixation.

iii) Discard the acetone and dry the plates and slides at room temperature.

iv) The plates can then be placed in a plastic bag, sealed and stored at –70°C until use. Chamber slides can be kept similarly in slide cases.

2.2.4. Preparation of serum dilutions

i) Dilute serum samples to a 1/20 dilution in PBS (0.01 M; pH 7.2) in separate 96-well plates (e.g. add 190 µl of PBS using a multichannel pipettor followed by 10 µl of the sera to be tested).

ii) Include as controls reference PRRSV antibody positive and negative sera of known titre.

2.2.5. Incubation of sera with fixed MARC-145 cells

i) Stored plates are removed from the –70°C freezer and when the plates reach room temperature rehydrate the cells with 150 µl PBS for a few minutes. Discard the PBS by inverting the plates and blotting dry on paper towels. Cells of eight-chamber slides are not rehydrated.

ii) Add volumes of 50 µl of each diluted serum to one well containing the fixed noninfected cells and to one well containing the fixed infected cells. Add similar volumes for each serum to a single chamber.

iii) Add volumes of 50 µl of the negative control serum and positive control serum dilutions in the same manner.

iv) Incubate the plates with their lids on at 37°C for 30 minutes in a humid atmosphere. Slides should be incubated similarly in boxes or slide trays with a cover.

v) Remove the serum samples and blot the plates dry on paper towels. A total of six washes using 200 µl of PBS are performed. The PBS is added to each well, followed by inversion of the plates to remove the PBS. After removing serum samples, slides are rinsed in PBS followed by a 10-minute wash.

2.2.6. Incubation with conjugate

i) Add volumes of 50 µl of appropriately diluted (in freshly prepared PBS) rabbit, mouse or goat anti-swine IgG (heavy and light chains) conjugated with FITC (fluorescein isothiocyanate) to each well using a multichannel pipettor. Similar volumes are added to individual chambers.

ii) Incubate plates or slides with their lids on at 37°C for 30 minutes in a humid atmosphere.

iii) Remove the conjugate from the plates and blot the plates dry on paper towels. A total of four washes using PBS are performed as described above. Discard the conjugate from the slides, rinse in PBS, wash for 10 minutes in PBS and rinse in distilled water. Tap the slides on an absorbent pad to remove excessive water.

iv) The plates and the slides are read using a fluorescence microscope.

2.2.7. Reading and interpreting the results

The presence of a green cytoplasmic fluorescence in infected cells combined with the absence of such a signal in noninfected cells is indicative of the presence of antibodies to PRRSV in the serum at the dilution tested. The degree of intensity of fluorescence may vary according to the amount of PRRSV-specific antibody present in the serum tested.

Absence of specific green fluorescence in both infected and noninfected cells is interpreted as absence of antibody to PRRSV in that serum at the dilution tested. The test should be repeated if the fluorescence is not seen with the use of the positive control sera on infected cells or if fluorescence is seen using the negative control serum on infected cells. No fluorescence should be seen on noninfected cells with any of the control sera. Any test serum giving suspicious results should be retested at a 1/20 dilution and if results are still unclear, a new serum sample from the same animal is requested for further testing.

2.3. Evaluation of sera for antibody titres by IFA

Microtitre plates and IFA may also be used for serum titration purposes. Up to 16 sera may be titred per 96-well microtitre plate.

2.3.1. Test procedure

i) Seed 96-well microtitre plates with MARC-145 cells (at a concentration 10^4 cells per a well) or PAM cells (approximately 10^5 cells per a well) and incubate at 37°C in a humidified 5% CO_2 incubator until they are confluent.

ii) Inoculate all wells with the PRRSV suspension at a concentration adjusted to produce approximately 100 foci of infected cells per a well (to facilitate correct reading of the results) except the wells of columns 1, 6 and 11. Incubate the plates at 37°C in a humidified 5% CO_2 incubator for 48–72 hours.

iii) Discard culture medium and rinse the monolayers once with PBS (0.01 M, pH 7.2). Fix the monolayers with cold acetone (80% aqueous solution) for 10 minutes at ambient temperature. Discard the acetone, air-dry the plates and keep the plates with lids at –20°C for short-term storage or –70°C for long-term storage, until use.

iv) Serially dilute sera including a PRRSV-positive control serum using a four-fold dilution in PBS, beginning at 1/16 or 1/20. Dilute a negative control serum at 1/16 or 1/20 dilution. Dispense 50 µl of each dilution (1/16, 1/64, 1/256, 1/1024 or 1/20, 1/80, 1/320, 1/1280) in wells containing viral antigen of columns 2, 3, 4, 5 or 7, 8, 9, 10. For each serum, also dispense 50 µl of dilution 1/16 or 1/20 in control wells of columns 1 and 6. Similarly dispense dilutions of positive and negative control sera in wells of columns 11 and 12.

v) Incubate the plates at 37°C for 30 minutes in a humid chamber. Discard the sera and rinse the plates three times using PBS.

vi) Add 50 µl of appropriately diluted anti-swine IgG conjugated with FITC and incubate plates at 37°C for 30 minutes in a humid chamber. Discard conjugate, rinse plates several times and tap the plates on absorbent material to remove excessive liquid.

2.3.2. Reading and interpreting the results

Following examination with a fluorescence microscope, the titre of a serum is recorded as the reciprocal of the highest serum dilution in which typical cytoplasmic fluorescence is observed. For paired serum samples, a four-fold increase in titre with a 2-week interval is indicative of active infection in an individual animal. No specific fluorescence should be observed with test sera or positive and negative control sera on noninfected control cells. No fluorescence should be seen on infected cells with negative control serum. Specific fluorescence should be observed on infected cells with positive control serum at appropriate dilutions. The IFA end-point may vary among laboratories. Test results may also vary depending on the PRRSV isolate used in the test because of antigenic diversity.

2.4. Detection of antibodies with the enzyme-linked immunosorbent assay

The ELISA is one of the most commonly used techniques for detection of antibodies specific to PRRSV, allowing fast, specific and sensitive confirmation of exposure to the virus. Several laboratories have developed ELISAs (indirect or blocking) for serological testing (Diaz *et al.*, 2012; Sorensen *et al.*, 1998; Venteo *et al.*, 2012). A double-blocking ELISA format that can distinguish between serological reactions to PRRSV-1 and PRRSV-2 species has been described (Sorensen *et al.*, 1998). Another study reported the development of an ELISA that allows differentiation of highly pathogenic PRRSV-2 from classical PRRSV-2 infections (Xiao *et al.*, 2014). ELISA kits are available commercially to determine the serological status of swine towards PRRSV, also in the oral fluids as a diagnostic matrix (Kittawornrat *et al.*, 2010; Venteo *et al.*, 2012). These kits use as antigens either one of the two species separately or combined antigens of both. Their main advantage is the rapid handling of a large number of samples. Commercial ELISAs are available that use mainly recombinant N proteins of both PRRSV species as antigens. The potential application of ELISAs based on the nonstructural proteins NSP1, NSP2 and NSP7 was also suggested. The performance of NSP7 ELISA was reported to be comparable to a commercial ELISA kit. Moreover, it allowed the differentiation of species-specific humoral response and resolved 98% of false-positive results of a commercial assay (Brown *et al.*, 2009). The ELISA method should be applied at the population level.

2.4.1. Antigen preparation and plate coating

For ELISAs directed at both species, mixed antigens derived from both PRRSV-1 and PRRSV-2 strains should be used. Preparation of purified recombinant antigens (e.g. recombinant nucleocapsid protein) is preferable and has been described (Chu *et al.*, 2009; Seuberlich *et al.*, 2002). Optionally, a high-quality antigen may be prepared by condensing an infected cell culture supernatant by a series of ultracentrifugation steps followed by solubilisation of the antigen pellet in 0.2% Triton X-100 (Cho *et al.*, 1996). A simpler and less expensive version of the ELISA based on antigens directly collected from a virus-infected primary culture of porcine alveolar macrophages (PAMs) is described here (Albina *et al.*, 1992). For PRRSV-2 strains MARC-145 or MA-104 continuous cell lines may also be used (Cho *et al.*, 1996).

i) Prepare PAM culture as described in Sections B.1.1.1 and B.1.1.2.

ii) Seed 15×10^6 macrophages onto a 25 cm^2 cell culture flask and incubate for 5 hours in RPMI 1640 medium supplemented with 10% FBS and 1% of an antibiotic-antimycotic solution under a humidified 5% CO_2 atmosphere at 37°C. Replace the medium to eliminate the non-attached cells and incubate for 20 hours.

iii) Add PRRSV isolate at an infection ratio of one viral particle for 40–80 cells.

iv) When the virus-induced cytopathic effect reaches a maximum, usually 3–4 days after the PRRSV infection, submit the flask to two or three freeze–thaw cycles.

v) Centrifuge at 800 *g* for 10 minutes, aliquot supernatant and freeze at –70°C until use.

vi) Mock antigen consisting of non-infected macrophage cell culture should be prepared in parallel.

vii) Determine the appropriate dilution of an antigen (previously inactivated by b-propiolactone treatment) to be used in ELISA by checkerboard titration: the positive antigen should be titrated in comparison with the mock antigen using the positive and negative control sera in order to determine the dilution giving the highest signal with the positive control and the lowest signal with the negative control.

viii) Determine the appropriate dilution of samples to be tested and the threshold value by preliminary testing of samples of known characteristics. Sera are diluted in saturation buffer (e.g. PBS supplemented with 10% FBS and 2% skim milk).

ix) Prepare the plates adding the positive and mock antigens appropriately diluted in PBS to alternative columns of a polystyrene 96-well microtiter plate, coat for 1 hour at 37°C and then overnight at 4°C.

2.4.2. Test procedure

i) Wash plates three times with PBS-Tween 20 (0.1%) for removal of the excess protein.

ii) Add 100 μl of the appropriate dilution of the sera to be tested to one positive and one mock antigen well.

iii) Add 100 μl of positive and negative control sera in duplicates to positive and mock antigen wells in parallel.

iv) Incubate plates at 37°C for 1 hour.

v) Wash three times in PBS-Tween 20.

vi) Add 100 μl of a rabbit anti-swine horseradish peroxidase conjugate to each well at the appropriate dilution, incubate for 30 minutes at 37°C.

vii) Wash plates 3 times in PBS-Tween 20.

viii) Add 100 μl of substrate solution (e.g. tetramethyl benzidine) to each well, incubate at room temperature for 15 minutes.

ix) Stop reaction by adding 50 μl of 1 N sulphuric acid.

x) Read optical density (OD) at a wavelength of 450 nm.

xi) Plate validation: Calculate ΔOD value for each sample, based on the difference between the positive antigen well OD minus the mock antigen well OD. ΔOD of the positive control serum minus the ΔOD of the negative control serum should be approximately > 0.5. Calculate the average ΔOD value for positive and negative controls. Depending on the accepted format, the results may be expressed as P/N ratio (positive antigen well OD/mock antigen well OD) or S/P value (ΔOD sample/ΔOD positive control).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Modified-live (MLV) and inactivated (killed) PRRSV vaccines are approved and commercially available in many countries for the control of the reproductive and/or respiratory forms of PRRS (Murtaugh & Genzow, 2011). It is assumed the most benefit from vaccination occurs when the vaccine virus is closely related antigenically to the field virus (Scortti *et al.,* 2006). However, there are no methods available to predict the vaccine efficacy. Although vaccination of pigs does not prevent PRRSV infection, it may be helpful in herds experiencing problems with PRRS. The killed vaccines are approved to be used as an aid in the reduction of abortions and weak piglets caused by the reproductive form of PRRS. MLV vaccines are intended to be used in sows and gilts 3–6 weeks prior to breeding and in piglets from 3 weeks of age or older (or in some cases down to one day old) as an aid in the reduction of diseases caused by PRRS.

MLV vaccines are not intended to be used in naive herds or boars of breeding age. Vaccine virus can persist in boars and be disseminated through semen (Christopher-Hennings *et al.,* 1997). MLV vaccine virus may be shed and transmitted to non-vaccinated contact pigs or vertically to offspring (Zimmerman *et al.,* 2019). Recombination events between vaccine strains or vaccine and wild-type strains have also been observed in the field (Kvisgaard *et al.,* 2020). Vaccines based on biotechnology are under development but not available on the market yet. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The isolate of PRRSV used for vaccine production must be accompanied by a history describing its origin and passage history. The master seed virus (MSV) must be safe in swine at the intended age of vaccination and provide protection against challenge. Isolates for a MLV vaccine must be shown not to revert to virulence after passage in host animals. It is recommended to determine the full genome sequence of the PRRS MSV. This reference sequence could then be used to control the genetic stability of the PRRS MLV during the production process or during the serial in vivo passages.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The MSV should be free from bacteria, fungi and mycoplasma. The MSV must be tested for and free from extraneous viruses, including transmissible gastroenteritis virus, porcine respiratory coronavirus, porcine epidemic diarrhoea virus, porcine adenovirus, porcine circovirus type 1 and 2, porcine haemagglutinating encephalitis virus, porcine parvovirus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic swine cell type. As a complement to culture, PCR could also be used to detect extraneous virus.

2.2. Method of manufacture

2.2.1. Procedure

PRRSV is propagated in a continuous cell line such as MARC-145 (clone of MA-104) cells. Viral propagation should not exceed five passages from the master seed virus (MSV) unless further passages prove to provide protection in swine.

The cell line is seeded into suitable vessels. MEM supplemented with FBS is used as the medium for production. Cell cultures are inoculated directly with PRRS working virus stock, which is generally from 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Killed virus vaccines are chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. MLV vaccines are generally mixed with a stabiliser before bottling and lyophilisation. If formalin is used as an inactivant, the final product should be tested for residual formaldehyde concentration, which should not exceed 0.74 g/litre.

2.2.2. Requirements for substrates and media

The FBS must be free from pestivirus and antibodies to pestivirus and free from bovine spongiform encephalopathy risk.

2.2.3. In-process control

Production lots of PRRSV for MLV and for inactivated (killed) virus vaccines must be titrated in tissue culture for standardisation of the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

2.2.4. Final product batch tests

Final container samples are tested for purity, safety and potency. MLV vials are also tested for the maximum allowable moisture content.

i)   Sterility and purity

Samples are examined for bacterial, fungal and pestivirus contamination. To test for bacteria in a MLV vaccine, ten vessels, each containing 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C for 14 days and observed for bacterial growth. To test for fungi, ten vessels, each containing 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C for 14 days and observed for fungal growth. Killed vaccines require 1.0 ml from ten final container samples be inoculated into the appropriate ten vessels of media. Pestivirus contamination should be evaluated according to the guidelines given in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and Chapter 3.9.2 *Classical swine fever*.

ii)   Safety

Target animal batch release safety tests or laboratory animal batch release safety tests should be avoided wherever possible. However, if animal-based batch release safety testing cannot be avoided, the testing should conform to the 3R principles. If regulatory authorities require such tests they can be conducted in a combination of guinea-pigs, mice or pigs.

iii)   Batch potency

Final container samples of an MLV vaccine are titrated ($\log_{10}$) in microtitre plates for determination of the titre.

a)   Test procedure

i)   Prepare tenfold dilutions from 10^{-1} through 10^{-5} by using 0.2 ml of rehydrated test vaccine and 1.8 ml of MEM. An internal positive control PRRSV should be titrated in the appropriate range.

ii)   Inoculate 0.1 ml/well from each dilution into five wells of a 96-well plate containing MARC-145 monolayers.

iii)   Incubate the plate at 37°C in a CO_2 atmosphere for 5–7 days.

iv)   Read the plates microscopically for CPE. The internal positive control PRRSV should give a titre within 0.3 $\log_{10}$ $TCID_{50}$ from its predetermined mean.

v)   Determine the $TCID_{50}$/dose by the Spearman–Kärber method. The release titre must be at least 1.2 logs higher than the titre used in the immunogenicity trial. The 1.2 logs include 0.5 logs for stability throughout the shelf life of the product and 0.7 logs for potency test variability.

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. Parallel-line assays using ELISA antigen-quantifying techniques to compare a standard with the final product are acceptable in determining the relative potency of a product. The standard should be shown to be protective in the host animal.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Field trial studies should be conducted to determine the safety of the vaccine. Non-vaccinated sentinel pigs should be included at each site for monitoring the shed of the attenuated virus.

ii) Reversion-to-virulence for attenuated/live vaccines

MSV must be shown not to revert to virulence after several passages in host animals, although the definition of virulence with such a virus is difficult. Attenuated PRRSV isolates are known to cause viraemia and will transmit to susceptible animals. The MSV should be shown to be avirulent in weaned piglets and pregnant animals by five serial passages (up to ten passages depending on country) of the MSV through susceptible swine using the most natural route of infection.

iii) Environmental consideration

Not applicable

2.3.2. Efficacy requirements

i) For animal production

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible swine against a virulent, unrelated challenge strain. For the respiratory form, 3-week-old piglets are vaccinated with the highest passage level of MSV. The piglets are challenged with approximately 10^5 TCID$_{50}$ a virulent isolate of PRRSV 2–16 weeks later to determine protection from respiratory clinical signs of PRRS. To determine protection from the losses caused by the reproductive form of PRRS, vaccinated animals are challenged at approximately 85 days' gestation. A prevented fraction, the proportion of potential PRRS disease occurrence reduced due to vaccination, is calculated to determine if there is acceptable protection, based on the proposed label claims, in the vaccinates from the clinical signs of reproductive disease, including fetal mummification, stillborn piglets or weak piglets, when compared with the controls. As MLV vaccines are frequently used in piglets bearing PRRSV specific maternally derived antibody (MDA), the interference of MDA with the efficacy of MLV vaccine should be evaluated.

Duration of immunity studies are conducted before the vaccine receives final approval. For the respiratory form of PRRS, duration should be shown up to the market age in pigs. Duration of immunity for the reproductive form should be shown through weaning of the piglets.

ii) For control and eradication

Not applicable

2.3.3. Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date.

Multiple batches of MLV vaccines should be re-titrated periodically throughout the shelf-life to determine vaccine variability. The release value should be adjusted if the titres are insufficient or highly variable.

Killed vaccines using *in-vivo* potency tests should be retested at expiry to demonstrate stability. Parallel-line assays using ELISA antigen-quantifying techniques should demonstrate the stability of the standard.

3. Vaccines based on biotechnology

Biotechnology-based vaccines are under development but not yet available on the market.

REFERENCES

ALBINA E., LEFORBAN Y., BARON T., PLANA DURAN J. & VANNIER P. (1992). An enzyme linked immunosorbent assay (ELISA) for the detection of antibodies to the porcine reproductive and respiratory syndrome (PRRS) virus. *Ann. Rech. Vet.*, **23**, 167–176.

BALKA G., PODGÓRSKA K., BRAR M.S., BÁLINT Á., CADAR D., CELER V., DÉNES L., DIRBAKOVA Z., JEDRYCZKO A., MÁRTON L., NOVOSEL D., PETROVIĆ T., SIRAKOV I., SZALAY D., TOPLAK I., LEUNG F.C. & STADEJEK T. (2018). Genetic diversity of PRRSV 1 in Central Eastern Europe in 1994–2014: origin and evolution of the virus in the region. *Sci. Rep.*, **8**, 7811.

BROWN E., LAWSON S., WELBON C., GNANANDARAJAH J., LI J., MURTAUGH M.P., NELSON E.A., MOLINA R.M., ZIMMERMAN J.J., ROWLAND R.R. & FANG Y. (2009). Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. *Clin. Vaccine Immunol.*, **16**, 628–635.

BIERNACKA K., PODGÓRSKA K., TYSZKA A. & STADEJEK T. (2018). Comparison of six commercial ELISAs for the detection of antibodies against porcine reproductive and respiratory syndrome virus (PRRSV) in field serum samples. *Res. Vet. Sci.*, **21**, 40–45.

CHRISTOPHER-HENNINGS J., NELSON E.A., NELSON J.K. & BENFIELD D.A. (1997). Effects of a modified-live virus vaccine against porcine reproductive and respiratory syndrome in boars. *Am. J. Vet. Res.*, **58**, 40–45.

CHO H.J., DEREGT D. &JOO H.S. (1996). An ELISA for porcine reproductive and respiratory syndrome: production of antigen of high quality. *Can. J. Vet. Res.*, **60**, 89–93.

CHU J.Q., HU X.M., KIM M.C., PARK C.S. & JUN M.H. (2009). Development and validation of a recombinant nucleocapsid protein-based ELISA for detection of the antibody to porcine reproductive and respiratory syndrome virus. *J Microbiol.*, **47**, 582–588.

DELRUE I., VAN GORP H., VAN DOORSSELAERE J., DELPUTTE P.L. & NAUWYNCK H.J. (2010). Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163. *BMC Biotechnol.*, **29**, 48.

DIAZ I., VENTEO A., REBOLLO B., MARTÍN-VALLS G.E., SIMON-GRIFÉ M., SANZ A. & MATEU E. (2012). Comparison of two commercial enzyme-linked immunosorbent assays for the diagnosis of porcine reproductive and respiratory syndrome virus infection. *J. Vet. Diagn. Invest.*, **24**, 344–348.

FENG L., ZHANG X., XIA X., LI Y., HE S. & SUN H. (2013). Generation and characterization of a porcine endometrial endothelial cell line susceptible to porcine reproductive and respiratory syndrome virus. *Virus Res.* **171**, 209–215.

GARCIA-NICOLAS O., BAUMANN A., VIELLE N.J., GÓMEZ-LAGUNA J., QUEREDA J.J., PALLARÉS F.J., RAMIS G., CARRASCO L. & SUMMERFIELD A. (2014). Virulence and genotype-associated infectivity of interferon-treated macrophages by porcine reproductive and respiratory syndrome viruses. *Virus Res.*, **179**, 204–211.

JUSA E.R., INABA Y., KOUNO M., HIROSE O., SHIBATA I., KUBOTA M. & YASUHARA H. (1996). Slow-reacting and complement-requiring neutralizing antibody in swine infected with porcine reproductive and respiratory (PRRS) virus. *J. Vet. Med. Sci.*, **58**, 749–753.

KARNIYCHUK U.U., GELDHOF M., VANHEE M., VAN DOORSSELAERE J., SAVELEVA T.A. & NAUWYNCK H.J. (2010). Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. *BMC Vet. Res.*, **4**, 30.

KARNIYCHUK U.U. & NAUWYNCK H.J. (2013). Pathogenesis and prevention of placental and transplacental porcine reproductive and respiratory syndrome virus infection. *Vet. Res.*, **44**, 95.

KITTAWORNRAT A., PRICKETT J., CHITTICK W., WANG C., ENGLE M., JOHNSON J., PATNAYAK D., SCHWARTZ T., WHITNEY D., OLSEN C., SCHWARTZ K. & ZIMMERMAN J. (2010). Porcine reproductive and respiratory syndrome virus (PRRSV) in serum and oral fluid samples from individual boars: will oral fluid replace serum for PRRSV surveillance? *Virus Res.*, **154**, 170–176.

KLEIBOEKER S.B., SCHOMMER S.K., LEE S.M., WATKINS S., CHITTICK W. & POLSON D. (2005). Simultaneous detection of North American and European porcine reproductive and respiratory syndrome virus using real-time quantitative reverse transcriptase-PCR. *J. Vet. Diagn. Invest.*, **17**,165–170.

KVISGAARD L.K, KRISTENSEN C.S., RYT-HANSEN P., PEDERSEN K., STADEJEK T., TREBBIEN R., ANDRESEN L.O. & LARSEN L.E. (2020). A recombination between two Type 1 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV-1) vaccine strains has caused severe outbreaks in Danish pigs. *Transbound. Emerg. Dis.*, doi: 10.1111/tbed.13555.

LALONDE C., PROVOST C. & GAGNON C.A. (2020). Whole-Genome Sequencing of Porcine Reproductive and Respiratory Syndrome Virus from Field Clinical Samples Improves the Genomic Surveillance of the Virus. *J. Clin. Microbiol.*, **58**, e00097-20.

MARTIN-VALLS G.E., KVISGAARD L.K., TELLO M., DARWICH L., CORTEY M., BURGARA-ESTRELLA A.J., HERNÁNDEZ J., LARSEN L.E. & MATEU E. (2014). Analysis of ORF5 and full-length genome sequences of porcine reproductive and respiratory syndrome virus isolates of genotypes 1 and 2 retrieved worldwide provides evidence that recombination is a common phenomenon and may produce mosaic isolates. *J. Virol.*, **6**, 3170–3181.

MORGAN S.B., GRAHAM S.P., SALGUERO F.J., SÁNCHEZ CORDÓN P.J., MOKHTAR H., REBEL J.M., WEESENDORP E., BODMAN-SMITH K.B., STEINBACH F. & FROSSARD J.P. (2013). Increased pathogenicity of European porcine reproductive and respiratory syndrome virus is associated with enhanced adaptive responses and viral clearance. *Vet. Microbiol.*, **163**, 13–22.

MURTAUGH M.P. & GENZOW M. (2011). Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). *Vaccine,* **26**, 8192–8204.

MURTAUGH M.P., STADEJEK T., ABRAHANTE J.E., LAM T.T. & LEUNG F.C. (2010). The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. *Virus Res.*, **154**, 18–30.

PROVOST C., JIA J.J., MUSIC N., LÉVESQUE C., LEBEL M.È., DEL CASTILLO J.R., JACQUES M. & GAGNON C.A. (2012). Identification of a new cell line permissive to porcine reproductive and respiratory syndrome virus infection and replication which is phenotypically distinct from MARC-145 cell line. *Virol. J.*, **13**, 267.

SCORTTI M., PRIETO C., MARTINEZ-LOBO F.J., SIMARRO I. & CASTRO J.M. (2006). Effects of two commercial European modified-live vaccines against porcine reproductive and respiratory syndrome viruses in pregnant gilts. *Vet. J.,* **172**, 506–514.

SEUBERLICH T., TRATSCHIN J.D., THÜR B. & HOFMANN M. (2002). Nucleocapsid protein-based enzyme-linked immunosorbent assay for detection and differentiation of antibodies against European and North American porcine reproductive and respiratory syndrome virus. *Clin. Diagn. Lab. Immunol.*, **9**, 1183–1191.

SHI M., LAM T.T., HON C.C., HUI R.K., FAABERG K.S., WENNBLOM T., MURTAUGH M.P., STADEJEK T. & LEUNG F.C. (2010a). Molecular epidemiology of PRRSV: a phylogenetic perspective. *Virus Res.*, **154**, 7–17.

SHI M., LAM T.T., HON C.C., MURTAUGH M.P., DAVIES P.R., HUI R.K., LI J., WONG L.T., YIP C.W., JIANG J.W. & LEUNG F.C. (2010b). Phylogeny-based evolutionary, demographic, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. *J. Virol.*, **84**, 8700–8711.

SORENSEN K.J., STRANDBYGAARD B., BØTNER A., MADSEN E.S., NIELSEN J. & HAVE P. (1998). Blocking ELISAs for the distinction between antibodies against European and American strains of porcine reproductive and respiratory syndrome virus. *Vet. Microbiol.,* **60**, 169–177.

STADEJEK T., LARSEN L.E., PODGÓRSKA K., BØTNER A., BOTTI S., DOLKA I., FABISIAK M., HEEGAARD P.M.H., HJULSAGER C.K., HUĆ T., KVISGAARD L.K., SAPIERZYŃSKI R. & NIELSEN J. (2017). Pathogenicity of three genetically diverse strains of PRRSV Type 1 in specific pathogen free pigs. *Vet. Microbiol.*, **209**,13–19.

STADEJEK T., OLEKSIEWICZ M.B., SCHERBAKOV A.V., TIMINA A.M., KRABBE J.S., CHABROS K. & POTAPCHUK D. (2008). Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. *Arch. Virol.,* **153**, 1479–1488.

STADEJEK T., STANKEVICIUS A., MURTAUGH M.P. & OLEKSIEWICZ M.B. (2013). Molecular evolution of PRRSV in Europe: current state of play. *Vet. Microbiol.*, **26**, 21–28.

TIAN K., YU X., ZHAO T., FENG Y., CAO Z., WANG C., HU Y., CHEN X., HU D., TIAN X., LIU D., ZHANG S., DENG X., DING Y., YANG L., ZHANG Y., XIAO H., QIAO M., WANG B., HOU L., WANG X., YANG X., KANG L., SUN M., JIN P., WANG S., KITAMURA Y., YAN J. & GAO G.F. (2007). Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS One*, **2**(6):e526.

TRUS I., BONCKAERT C., VAN DER MEULEN K. & NAUWYNCK H.J. (2014). Efficacy of an attenuated European subtype 1 porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs upon challenge with the East European subtype 3 PRRSV strain Lena. *Vaccine,* **32**, 2995–3003.

VENTEO A., REBOLLO B., SARRASECA J., RODRIGUEZ M.J. & SANZ A.(2012). A novel double recognition enzyme-linked immunosorbent assay based on the nucleocapsid protein for early detection of European porcine reproductive and respiratory syndrome virus infection. *J. Virol. Methods.,* **181**, 109–113.

WEESENDORP E., MORGAN S., STOCKHOFE-ZURWIEDEN N., POPMA-DE GRAAF D.J., GRAHAM S.P. & REBEL J.M. (2013). Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. *Vet. Microbiol.*, **12**, 1–12.

WERNIKE K., BONILAURI P., DAUBER M., ERRINGTON J., LEBLANC N., REVILLA-FERNÁNDEZ S., HJULSAGER C., ISAKSSON M., STADEJEK T., BEER M. & HOFFMANN B. (2012a). Porcine reproductive and respiratory syndrome virus: interlaboratory ring trial to evaluate real-time reverse transcription polymerase chain reaction detection methods. *J. Vet. Diagn. Invest.*, **24**, 855–866.

WERNIKE K., HOFFMANN B., DAUBER M., LANGE E., SCHIRRMEIER H. & BEER M. (2012b). Detection and typing of highly pathogenic porcine reproductive and respiratory syndrome virus by multiplex real-time rt-PCR. *PLoS One.,* **7**, 38251.

XIAO Y.H., WANG T.T., ZHAO Q., WANG C.B., LV J.H., NIE L., GAO J.M., MA X.C., HSU W.H. & ZHOU E.M. (2014). Development of Indirect ELISAs for Differential Serodiagnosis of Classical and Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus. *Transbound. Emerg. Dis.*, **61**, 341–349.

YOON I.J., JOO H.S., CHRISTIANSON W.T., KIM H.S., COLLINS J.E., MORRISON R.B. & DIAL G.D. (1992). An indirect fluorescent antibody test for the detection of antibody to swine infertility and respiratory syndrome virus in swine sera. *J. Vet. Diagn. Invest.,* **4**, 144–147.

ZHOU L., ZHANG J., ZENG J., YIN S., LI Y., ZHENG L., GUO., GE X. & YANG H. (2009) The 30-amino-acid deletion in the Nsp2 of highly pathogenic porcine reproductive and respiratory syndrome virus emerging in China is not related to its virulence. *J. Virol.,* **83**, 5156–5167.

ZHOU L. & YANG H. (2010). Porcine reproductive and respiratory syndrome in China. *Virus Res.* **154**, 31–37.

ZIMMERMANN J.J., DEE S.A., HOLTKAMP D.J., MURTAUGH M.P., STADEJEK T., STEVENSON G.W., TORREMOREL M., YANG H. & ZHANG J. (2019). Porcine Reproductive and Respiratory Syndrome Viruses (Porcine Arteriviruses). *In:* Diseases of Swine, Eleventh Edition, Zimmerman J.J., Karriker L.A., Ramirez A., Schwartz K.J., Stevenson G.W., Zhang J. eds., Wiley-Blackwell; USA, 685–708.

*

* *

NB: There are WOAH Reference Laboratories for porcine reproductive and respiratory syndrome
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for porcine reproductive and respiratory syndrome

NB: FIRST ADOPTED IN 1996; MOST RECENT UPDATES ADOPTED IN 2021.