CHAPTER 2.8.7.

PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

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, a virus currently classified as a member of the order Nidovirales, family Arteriviridae, genus Arterivirus. The primary target cell of the virus is the alveolar macrophage of the pig. Two major antigenic types of the virus exist, the type 1 (European) and the type 2 (American). The virus is primarily transmitted via infected pigs but also by faeces, urine, semen and fomites.

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. No other species are known to be naturally infected with PRRSV.

Identification of the agent: Virological diagnosis of PRRS virus infection is difficult; the virus can be isolated from tissues such as serum, ascitic fluids, or organ samples, such as lungs, tonsils, lymph nodes and spleen of affected pigs. As porcine alveolar macrophages are the most susceptible culture system for virus of both antigenic types, these cells are recommended for virus isolation. MARC-145 (MA-104 clone) cells are also suitable. There is variability between batches of macrophages in their susceptibility to PRRS virus. Thus, it is necessary to identify a batch with high susceptibility, and maintain this stock under liquid nitrogen until required. The virus is identified and characterised by immunostaining with specific antisera. Additional techniques, such as immunohistochemistry and in situ hybridisation on fixed tissues and reverse-transcription polymerase chain reaction, have been developed for laboratory confirmation of PRRS virus infection.

Serological tests: A wide range of serological tests is currently available for the detection of serum antibodies to PRRS virus. The immunoperoxidase monolayer assay uses alveolar macrophages and the indirect immunofluorescence assay uses MARC-145 cells that are usually infected using either the European or the American antigenic type of the virus, respectively. Both assays can however be designed with both cell and PRRS virus types. Commercial or in-house enzyme linked immunosorbent assays (ELISA) are now often used. Several commercial ELISAs are specific for both the European and American types of the virus. An indirect ELISA, a blocking ELISA and a double ELISA that can distinguish between serological reactions to the European and the American types have been described.

Requirements for vaccines: Vaccines can be of value as an aid in the prevention of reproductive and respiratory forms of PRRS. Modified live vaccines are not suitable for use in pregnant sows and gilts and in boars. Vaccination may result in shedding of vaccinal virus in semen. Modified live virus vaccines can persist in vaccinated animals, and transmission to nonvaccinated animals and subsequent vaccine-virus-induced disease have been reported.

A. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is characterised by reproductive failure of sows and respiratory disease in pigs. (Benfield et al., 1999). The disease was first recognised in 1987 in the United States of America, and within a few years it became a pandemic. PRRS is caused by the PRRS virus (PRRSV). It was...
discovered in 1991 in The Netherlands (Wensvoort et al., 1993) and is classified as a member of the order Nidovirales, family Arteriviridae, genus Arterivirus (Brinton et al., 2000). PRRSV is a single-stranded positive-sense RNA virus and the biology of the virus has been well characterised. Soon after the discovery of the virus it became apparent the North American (NA, type 2) and European (EU, type 1) PRRSV isolates represented two genotypes with antigenic differences (Larochelle & Magar, 1997c; Magar et al., 1997). Additional investigations have demonstrated regional differences within each continent. These differences are now becoming blurred as type 2 PRRSV has been introduced into Europe (in part through the use of a modified-live vaccine made from a North American isolate) and type 1 virus has been discovered in North America. Most PRRSV isolates from South America and much of Asia are of type 2 and it is assumed these viruses were introduced through the movement of swine and/or semen. A reportedly highly virulent strain of type 2 virus in Southeast Asia is characterised by a discontinuous 30 amino acid deletion in the NSP2 region of the genome.

There is an increasing diversity among strains of the two genotypes, which has been attributed to the high error-rate inherent in PRRSV replication (Chang et al., 2002) and recombination between strains (Van Vugt et al., 2001). There have also been recent descriptions of East European strains of type 1 PRRSV with a high degree of polymorphism, providing further insights into the emergence of the relatively new pathogen of pigs (Stadejek et al., 2006; 2008). The effects of such diversity on diagnostics and vaccines are largely unknown, but do raise concerns and should be considered.

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. 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 infected pigs and also by faeces, urine and semen. It can also be spread indirectly, presumably via aerosol routes, leading to chronic re-infection of herds in swine dense areas, and possibly by mechanical vectors. Gross and microscopic lesions consistent with PRRSV infection have been well described (Halbur et al., 1995). 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 are believed to exist based on field observations and some experimental studies (Halbur et al., 1995). 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

#### 1. Identification 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 lavage ideal samples to collect for detection of PRRSV. Isolation of PRRSV can be difficult as not all virus isolates (especially type 1 viruses) can easily infect a cell line derived from the MA-104 monkey kidney cell line (Kim et al., 1993). Interestingly, this continuous cell culture system has been the only one reported to sustain a PRRSV infection. Porcine alveolar macrophages (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 source of PAM (Wensvoort et al., 1993). Different batches of PAM are not always equally susceptible to PRRSV; thus it is 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. Detection of PRRSV nucleic acid can be accomplished with reverse-transcription polymerase chain reaction (RT-PCR), nested set RT-PCR, and real-time RT-PCR (Drew, 1995b; Kleiboeker et al., 2003; Larochelle & Magar, 1997a; Mardassi et al., 1994; Wasilk et al., 2004). These tests are commonly used to detect nucleic acid in tissues and serum. They 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. A multiplex PCR assay has been designed to differentiate isolates of types 1 and 2 (Gilbert et al., 1997). Restriction fragment length polymorphism analysis of PCR-amplified products has been developed for the differentiation of field and vaccine PRRSV isolates (Wesley et al., 1998) and recently molecular epidemiological studies of PRRSV strains have been performed using phylogenetic analyses of specific structural gene sequences. All of these nucleic acid tests are more rapid than virus isolation and do not require cell culture.
infrastructure. Although seldom used for diagnostic purposes, in-situ hybridisation is capable of detecting and differentiating type 1 and 2 PRRSV genotypes in formalin-fixed tissues (Laroche & Magar, 1997c). The sensitivity and specificity of the methods for detection of PRRSV genome can be compromised by the very high genetic diversity of PRRSV, especially in type 1. Immunohistochemistry can be used to identify viral proteins (Halbur et al., 1994; Laroche & Magar, 1995) and when performed on formalin-fixed tissues enables the visualisation of antigen together with histological lesions.

**a) Harvesting of alveolar macrophages from lungs**

Lungs should preferably be obtained from SPF 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 1000 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 $6 \times 10^7$ macrophages/1.5 ml. Macrophage batches should not be mixed.

**b) 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). It is recommended that alveolar macrophages and fetal bovine serum (FBS) to supplement culture medium be pestivirus free.

**c) Virus isolation 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 ascitic fluids, 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 $6 \times 10^7$ 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 (Rose-Peake Memorial Institute) 1640 medium supplemented with 5% FBS and 10% 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, ascitic fluid, 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 (1/10,000 dilution). Shake the plates and transfer 10 µl from rows C and G to rows D and H (1/1,000,000 dilution). Shake the plates.

**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 antisera or MAb.

v) **Immunostaining with a PRRSV-positive antisera or MAb**

Infect macrophages with 50 μl of supernatant or tissue sample as described in Section B.2.a, 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.a or B.2.b.

### 2. Serological tests

A variety of assays for the detection of serum antibodies to PRRSV have been described. Serological diagnosis is, in general, easy to perform, with good specificity and sensitivity, especially on a herd basis. Sera of individual pigs sometimes cause difficulties because of nonspecific reactions, but this problem may be solved by resampling the pig after 2–3 weeks. Serology is generally performed with a binding assay, such as the IPMA, immunofluorescence assay, or the enzyme-linked immunosorbent assay (ELISA) – of which many varieties are described (Albina et al., 1992; Cho et al., 1997; Denac et al., 1997; Houben et al., 1995; Jusa et al., 1996; Nodelijk et al., 1996; Wensing et al., 1993; Yoon et al., 1992). These tests are often performed with viral antigen of one antigenic type, which means that antibodies directed against the other, heterologous, antigenic type 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 types 1 and 2 virus as antigen and thus it can distinguish between serological reaction to both types (Sorensen et al., 1998). The first live attenuated vaccine for PRRS based on type 2 virus has been observed to spread to nonvaccinated animals (Botner et al., 1997; Torrison et al., 1996), and subsequent development in herds of vaccine-virus-induced PRRS reproductive failures has been reported in Denmark (Botner et al., 1997; Madsen et al., 1998). Reaction to type 2 vaccine-like PRRSV can be anticipated in countries using or having used this vaccine; European countries may therefore observe reactions and isolation of both antigenic types (Botner et al., 1997; Madsen et al., 1998). The identification of type 1 strains of PRRSV in the USA and Canada has also been reported (Fang et al., 2004), but the prevalence of infection by such strains is not well documented. As both types of PRRSV are globally spread, serological tests should contain antigens of both types.

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 meat juice and oral fluid. Neutralising antibodies develop slowly and do not reach high titres. They can be detected from 3 to 4 weeks after infection and they can persist for 1 year or more. The use of complement to make the serum virus neutralisation test more sensitive has been reported (Dea 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 antigenic types. This assay can also be adapted to the MARC-145 cell line for both the European and American types (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. Commercial ELISAs with good sensitivity and specificity are available and have been compared (Drew, 1995a).

#### a) 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 object 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 duplicate. Test sera are diluted and incubated on the cell substrate. If antibodies are present in the test 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 solution. Reading of the test is done with an inverted microscope.

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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)
• **Seeding macrophages in the microtitre plates**
  i) Defrost one vial containing $6 \times 10^7$ 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 5% FBS, 100 IU (International Units) penicillin and 100 \( \mu \)g streptomycin (growth medium).
  iv) Dispense 100 \( \mu \)l 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-ethanesulphonic acid) in the medium.

• **Infection of cells with PRRSV**
  i) Add to each well 50 \( \mu \)l 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.

• **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.

• **Preparation of serum dilutions in a dilution plate**
  i) Dispense 180 \( \mu \)l 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 \( \mu \)l of dilution buffer to all other wells.
  iii) Add 20 \( \mu \)l 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 \( \mu \)l 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.

• **Incubation of sera in the plate with fixed macrophages**
  i) Transfer 50 \( \mu \)l 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.

• **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 \( \mu \)l 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.

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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 \( \mu \)l 30% \( H_2O_2 \) for each 20 ml of chromogen/substrate solution.

Filter the solution through a 5 \( \mu \)m filter.
Staining procedure
i) Dispense 50 µl of the filtered chromogen/substrate (AEC) solution to all wells of the plate(s) (see footnote 1).
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 1).

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.

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 b1 below).

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 penicillin and 100 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% CO₂ incubator to obtain a monolayer with approximately 40–50% of the cells infected as determined by indirect immunoﬂuorescence. 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 CO₂ incubators are not available.

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% CO₂ 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% CO₂ incubator. At this time 15–20 infected cells per field of view may be observed by indirect immunofluorescence.

- **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.

- **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.

- **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.

- **Incubation with conjugate**
  i) Add volumes of 50 µl of appropriately diluted (in freshly prepared PBS) rabbit 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.

- **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.

b1) Evaluation by IFA of sera for antibody titres

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

i) Seed 96-well microtitre plates with MARC-145 cells and incubate at 37°C in a humidified 5% CO₂ incubator until they are confluent.

ii) Inoculate all wells with the PRRSV preparation except the wells of columns 1, 6 and 11, and incubate the plates at 37°C in a humidified 5% CO₂ 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.

• 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.

c) Detection of antibodies with the enzyme-linked immunosorbent assay

Several laboratories have developed ELISAs (indirect or blocking) for serological testing (Albina et al. 1992; Cho et al., 1997; Denac et al., 1997; Houben et al., 1995; Sorensen et al., 1998). A double-blocking ELISA format that can distinguish between serological reactions to the European and the American antigenic type has been described (Sorensen et al., 1998). ELISA kits are available commercially to determine the serological status of swine towards PRRSV. These kits use as antigens either the European or the American PRRSV types separately or as combined antigens. Their main advantage is the rapid handling of a large number of samples. Commercial ELISAs are available that use recombinant proteins of both PRRSV types as antigens.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

Modified-live (MLV) PRRSV vaccines are commercially available in many countries for the control of the reproductive and/or respiratory forms of PRRS. In the USA and Europe, a killed virus vaccine is licensed as an aid in the reduction of abortions and weak piglets caused by the reproductive form of PRRS. All PRRS vaccines currently licensed in the USA contain the antigenic type 2. In Europe, three MLV vaccines are licensed and available commercially: one is based on antigenic type 2 and two others are type 1. It is
assumed the most benefit from vaccination occurs when the vaccine virus is more closely related antigenically to the field virus (Larochelle & Magar, 1997b; Scortti et al., 2006). Although vaccination of pigs does not prevent PRRSV infection, it may be helpful in herds experiencing problems with PRRS or herds at high risk of PRRSV infection. MLV vaccines are not intended to be used in naive herds, pregnant sows or gilts or boars of breeding age. 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 as an aid in the reduction of diseases caused by PRRS. 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 nonvaccinated contact pigs (Torrison et al., 1996).

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

a) Characteristics of the seed
   i) 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 not revert to virulence after passage in host animals.

   ii) 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 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.

b) Method of manufacture
   i) Procedure
   The PRRSV is propagated in a continuous African green monkey kidney cell line, such as MA-104 or Vero. Viral propagation should not exceed five passages from the master seed virus (MSV) unless further passages prove to provide protection in swine.

   The African green monkey kidney 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 and should not exceed 0.74 g/litre.

   ii) Requirements for substrates and media
   The FBS must be free from pestivirus or antibodies to pestivirus and free from bovine spongiform encephalopathy risk.

   iii) In-process control
   Production lots of PRRSV 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.

   iv) 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.

   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.

**Safety**

Safety tests can be conducted in a combination of guinea-pigs, mice or pigs.

**Batch potency**

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

- **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) Incubate 0.1 ml/well from each dilution into five wells of a 96-well plate containing African green monkey kidney monolayers.

  iii) 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.

  iv) 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.

c) **Requirements for authorisation**

i) **Safety requirements**

  **Target and non-target animal safety**

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

  **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.

  **Environmental consideration**

  Not applicable

ii) **Efficacy requirements**

  **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 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 and/or weak piglets, when compared with the controls.
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.

*For control and eradication*

Not applicable

iii) 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

a) Vaccines available and their advantages

None.

b) Special requirements for biotechnological vaccines, if any

None.

REFERENCES


Chapter 2.8.7. — Porcine reproductive and respiratory syndrome


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NB: There is an OIE Reference Laboratory for Porcine reproductive and respiratory syndrome (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).