

SWINE VESICULAR DISEASE

SUMMARY

Swine vesicular disease (SVD) is a contagious disease of pigs, caused by an enterovirus and characterised by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. Strains of SVD virus may vary in virulence, and the disease may be subclinical, mild or severe, the latter usually only being seen when pigs are housed on abrasive floors in damp conditions. The main importance of SVD is that it is clinically indistinguishable from foot and mouth disease (FMD), and any outbreaks of vesicular disease in pigs must be assumed to be FMD until investigated by laboratory tests and proven otherwise. However, subclinical infection has been the most frequent condition observed during recent years.

Identification of the agent: Where a vesicular condition is seen in pigs, the demonstration by enzyme-linked immunosorbent assay (ELISA) of SVD viral antigen in a sample of lesion material or vesicular fluid is sufficient for a positive diagnosis. If the quantity of lesion material submitted is not sufficient (less than 0.5 g), or if the test results are negative or inconclusive, a more sensitive test, such as the reverse transcription polymerase chain reaction (RT-PCR) or isolation of virus (VI) in porcine cell cultures, may be used. If any inoculated cultures subsequently develop a cytopathic effect, the demonstration of SVD viral antigen by ELISA or by RT-PCR will suffice to make a positive diagnosis. Subclinical infection may be detected by random sampling of pen-floor faeces followed by identification of SVD viral genome using RT-PCR or VI tests.

Serological tests: Serological tests can be used to help confirm clinical cases as well as to identify subclinical infections. Specific antibody to SVD virus can be identified using the microneutralisation test or ELISA. Although the microneutralisation test requires 2–3 days to complete, it remains the definitive test for antibody to SVD virus. A small proportion (up to 0.1%) of normal, uninfected pigs will react positively in serological tests for SVD. The reactivity of these singleton reactors is transient, so that they can be differentiated from infected pigs by resampling of the positive animal and its cohorts.

Requirements for vaccines and diagnostic biologicals: There are currently no commercial vaccines available against SVD. Diagnostic and standard reagents are available from regional reference laboratories.

A. INTRODUCTION

Swine vesicular disease (SVD) can be a subclinical, mild or severe vesicular condition depending on the strain of virus involved, the route and dose of infection, and the husbandry conditions under which the pigs are kept. Clinically, SVD is indistinguishable from foot and mouth disease (FMD) and this is its main importance. It is therefore urgent that cases of SVD be distinguished from FMD by laboratory investigation. Recent outbreaks of SVD have been characterised by less severe, or no clinical signs; infection has been detected when samples are tested for a serosurveillance programme or for export certification.

The incubation period for SVD is between 2 and 7 days, after which a transient fever of up to 41°C may occur. Vesicles then develop on the coronary band, typically at the junction with the heel. These may affect the whole coronary band resulting in loss of the hoof. More rarely, vesicles may also appear on the snout, particularly on the dorsal surface, on the lips, tongue and teats, and shallow erosions may be seen on the knees. Affected pigs may be lame and off their feed for a few days. Abortion is not a typical feature of SVD. Recovery is usually complete in 2–3 weeks, with the only evidence of infection being a dark, horizontal line on the hoof where growth has been temporarily interrupted. The clinical signs vary according to the age of pigs affected, the conditions under which they are kept and the strain of SVD virus involved (13). Disease caused by mild strains may remain unobserved, particularly in pigs kept on grass or housed on deep straw. Younger animals are more severely affected, although

mortality due to SVD is very rare, in contrast with FMD in young stock. Nervous signs have been reported, but are unusual. Affected pigs may excrete virus from the nose and mouth and in the faeces up to 48 hours before the onset of clinical signs. Most virus is produced in the first 7 days after infection, and virus excretion from the nose and mouth normally stops within 2 weeks. Virus may continue to be shed for up to 3 months in the faeces. The SVD virus is extremely resistant to inactivation in the environment, and is stable in the pH range 2.5–12.0 (14). This is in contrast to the FMD virus, which is very labile outside the pH range 6.0–8.0.

Because SVD may be mild or subclinical, it is essential when submitting samples from suspect clinical cases that serum samples from both the suspect pigs and other apparently unaffected animals in the group be included. It is possible for SVD to circulate unnoticed until it affects a particularly susceptible group, and therefore, in order to ascertain how long infection has been present, it is necessary to look for seroconversion to SVD virus in apparently healthy animals. Also the identification of the isotype of the immunoglobulins (M or G) to SVD virus may help to ascertain the time of exposure to infection.

SVD is clinically very similar to FMD. Samples for virus isolation or antigen detection must be handled and submitted as though they contained FMD virus and must be transported in phosphate buffered saline (PBS) mixed with glycerol (1/1), pH 7.2–7.6, with antibiotics such as (final concentration per ml) penicillin (1000 International Units [IU]), neomycin sulphate (100 IU), polymyxin B sulphate (50 IU), and mycostatin (100 IU) (10).

SVD virus has been classified as a pig enterovirus, in the family Picornaviridae. All isolates are classified in a single serotype, with four distinguishable antigenic/genomic variants (2). Antigenically, SVD virus is related to the human virus coxsackievirus B5. There are reports of seroconversion to SVD virus in laboratory workers handling the agent. Clinical disease was reported to be mild with the exception of a single case of meningitis associated with SVD virus infection. However, there have been no reported cases of seroconversion or disease in farmers or veterinarians working with infected pigs. Under experimental conditions, it has not been possible to show transmission of coxsackievirus B5 between pigs.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Any vesicular condition in pigs may be FMD. Once suspicion of FMD has been eliminated, the diagnosis of SVD requires the facilities of a specialised laboratory. Countries that lack such a facility should send samples for investigation to an OIE Reference Laboratory for SVD¹. In the Americas, parallel testing for vesicular stomatitis viral antigen should also be conducted.

The detection of antigens or genome of SVD virus by means of enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) has the same diagnostic value as virus isolation. Due to their speed, ELISA and RT-PCR make suitable screening tests. However, virus isolation is the reference method and should be used if a positive ELISA or RT-PCR result is not associated with the detection of clinical signs of disease, the detection of seropositive pigs, or a direct epidemiological connection with a confirmed outbreak.

If there are clinical signs, investigation should start with the examination of a 10% suspension of lesion material in PBS. Faecal samples are the specimen of choice for the detection of virus where subclinical SVD is suspected. Faecal samples can be collected from individual pigs or from the floor of premises suspected to contain, or to have contained, pigs infected with SVD. The level of virus in faeces is usually insufficient for detection by ELISA and the use of RT-PCR and/or virus isolation is required. A significant proportion of faecal samples inoculated into cell cultures will give rise to the growth of other enteroviruses. These can be differentiated from SVD virus by ELISA or RT-PCR, but they may also outgrow SVD virus that is present, and give rise to false negative results.

o Preparation of samples

Lesion material: a suspension is prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium and antibiotics. Further medium should be added to obtain approximately a 10% suspension. This is clarified by centrifugation at 10000 rpm for 20–30 minutes in a high speed centrifuge and the supernatant is harvested.

Faecal samples: faecal material (approximately 20 g) is resuspended in a minimal amount of tissue culture medium or phosphate buffer (0.04 M phosphate buffer or PBS). The suspension is homogenised by

1 FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD, Institute for Animal Health, Pirbright, Woking, Surrey GU24 0NF, United Kingdom (also an OIE Reference Laboratory for FMD and SVD). Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Brescia, Via Bianchi 9, Italy.

vortexing and clarified by centrifugation at 10000 rpm for 20–30 minutes in a high speed centrifuge; the supernatant is harvested and filtered through 0.45 µm filter.

a) Virus isolation

A portion of the clarified epithelial or faecal suspension is inoculated on to monolayers of IB-RS-2 cells or other susceptible porcine cells, grown in appropriate containers (25 cm² flasks, rolling tubes, 24-, 12-, 6-well plates). For differential diagnosis (e.g. FMD) in case of clinical lesion bovine cell culture systems should also be employed. Generally SVD virus will grow in cells of porcine origin only, however there is a report that the virus can be isolated in secondary lamb kidney cells. Tissue culture medium is supplemented with, 10% bovine serum for cell growth, with 3% bovine serum for maintenance, and with antibiotics.

Cultures are examined daily. If a cytopathic effect (CPE) is observed, the supernatant fluid is harvested and virus identification is performed by ELISA (or other appropriate test, e.g. RT-PCR). Negative cultures are blind-passaged after 48 or 72 hours, and observed for a further 2–3 days. If no CPE is evident after the second passage, the sample is recorded "NVD" (no virus detected). When isolating virus from faeces in which the amount of virus present may be low, a third tissue culture passage may be required.

b) Immunological methods

• **Enzyme-linked immunosorbent assay**

The detection of SVD viral antigen by an indirect sandwich ELISA has replaced the complement fixation test as the method of choice. The test is the same as that used for FMD diagnosis. Wells of ELISA plates are coated with rabbit antiserum to SVD virus. This is the capture serum. Test sample suspensions are added and incubated. Appropriate controls are also included. Guinea-pig detection serum is added at the next stage followed by rabbit anti-guinea-pig serum conjugated to horseradish peroxidase. Extensive washing is carried out between each stage to remove unbound reagents. A positive reaction is indicated if there is a colour reaction on the addition of chromogen (for example orthophenylenediamine) and substrate (H₂O₂). With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at the appropriate wavelength, in which case an absorbance reading ≥0.1 above background indicates a positive reaction. As an alternative to guinea-pig and rabbit antisera, suitable monoclonal antibodies (MAbs) can be used, coated to the ELISA plate as the capture antibody, or peroxidase conjugated as tracing antibody.

An MAb-based ELISA can also be used to study antigenic variation among strains of SVD virus. Tissue-culture grown viral strains are trapped by a rabbit hyperimmune antiserum to SVD virus adsorbed to the solid phase. Appropriate panels of MAbs are then reacted and the binding of MAbs to field strains is compared with the binding of MAbs to the parental strains. Strong binding indicates the presence of epitopes shared between the parental and the field strains (2).

c) Nucleic acid recognition methods

Reverse transcription followed by the PCR (RT-PCR) is a useful method to detect SVD viral genome in a variety of samples from clinical and subclinical cases. Several methods have been described (3, 7, 12, 15–18), employing different techniques for RNA extraction, targeting different parts of the SVD virus genome and using different approaches to detect the DNA products of amplification. In the case of faecal samples, an immunocapture technique using a SVD virus-specific MAb has been shown to be effective (7). This method (described below) is suitable for laboratories without sophisticated equipment for real-time detection of DNA amplification products, but where such facilities are available an approach such as that described by Reid *et al.* (16, 17) offers advantages in terms of ease of use and reduced risk of laboratory contamination by PCR products.

- i) RNA Immune-extraction: Coat wells of an ELISA plate with a saturating solution of MAb 5B7 (200 µl/well, diluted in carbonate-bicarbonate buffer) by overnight incubation at 4°C. Wash plates three times with PBS. Use plates immediately or store at –20°C for up to 2–3 weeks, or more if stabilised. NOTE: As an alternative to immunocapture, RNA extraction can be performed using a suitable commercially available kit (such as RNeasy kit from Qiagen).
- ii) Distribute each sample (faeces suspension) into three wells of the 5B7-coated plate (200 µl/well, 600 µl of sample in total).
- iii) After incubation for 1 hour at 37°C with very slow shaking, wash wells three times with PBS. Washing is performed manually, in order to avoid cross-contamination between wells.
- iv) RNA is extracted from each sample by adding approximately 100 µl/well of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.5% Sarkosyl). Incubate wells for 3–5 minutes and recover the sample from the three wells (300–350 µl total), and transfer into a single tube.

- v) RNA is then precipitated by adding a mixture of 750 µl of absolute ethanol and 35 µl of 3 M sodium acetate (pH 5.2); vials are vortexed and incubated at -20°C for a minimum of 1 hour (prolonged overnight precipitation at -20°C may also be suitable).
- vi) Centrifuge the sample at 13,000 rpm for 30 minutes at 4°C, after which a pellet should be visible which should be washed with 500 µl of 70% cold ethanol (centrifuged at 13000 rpm for 10 minutes at 4°C) and dried.
- vii) Assemble the reaction mixes for the reverse transcription of SVDV RNA. Mixes (20 µl total volume) contain 4 µl of 5× AMV reaction buffer (final reaction concentration is 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol, 50 mM Tris/HCl, pH 8.5), 2 µl 10 mM dNTP mix, 1 µl Random Primer pd(N)₆ (100 pmol), 0.3 µl RNase Inhibitor (12 units), 0.2 µl AMV reverse transcriptase (5 units) 0.5 µl BSA (0.5 µg) and 12 µl of nuclease-free water.
- viii) Resuspend the RNA pellet in 20 µl of reverse transcription mix (see above).
- ix) Incubate reactions at 42°C for 60 minutes followed by 95°C for 3 minutes.
- x) PCR for SVDV Genome: Add 5 µl of prepared cDNA to 20 µl PCR amplification mix containing 0.55 µl 2M KCl (final reaction concentration is 44 mM), 2 µl 10 mM dNTP mix, 1 µl pSVDV-SA2 rev primer (10 pmol: 5'-TCA-CGT-TTG-TCC-AGG-TTA-CC-3'), 1 µl pSVDV-SS4 dir primer (10 pmol: 5'-TTC-AGA-ATG-ATT-GCA-TAT-GGG-G-3'), 0.25 µl Taq polymerase (1.24 units) and 15.2 µl of nuclease-free water.
- xi) Place the plate in a PCR thermocycler and run the following programme:
One cycle at 94°C for 3 minutes,
40 Cycles of 94°C for 20 seconds, 60°C for 20 seconds, 72°C for 30 seconds,
One cycle at 72°C for 5 minutes.
- xii) Mix a 20 µl aliquot of each sample with 4 µl of staining solution and load onto a 2% agarose gel. After electrophoresis, a positive result is indicated by the presence of a 154 bp fragment of SVDV RNA polymerase (3D) gene in the gel.

Comparative analysis of sequences of the viral genome is useful to establish relationships between isolates of SVD virus. By sequencing the *1D* gene, which codes for the major structural protein VP1, it has been possible to group strains of SVD virus according to their sequence homology, and to relate epidemiologically strains causing disease in different regions or at different times (2). A database of ID gene sequences of SVD viruses is held at the OIE Reference Laboratory, Pirbright, UK.

2. Serological tests

These are used in the laboratory confirmation of outbreaks, for serological surveillance and for export certification of pigs. SVD is often diagnosed solely on the evidence of serological tests. Because of the subclinical or mild nature of the disease, it is often first suspected following routine serology for disease surveillance or export certification. The virus neutralisation (VN) test, the double immunodiffusion test, the radial immunodiffusion test, the counter immunoelectrophoresis test and the ELISA have all been described for the detection of antibodies to SVD virus (1, 5, 8). However, the VN test and the ELISA are the only techniques commonly used. The VN test is the accepted standard test, but has the disadvantage that it takes 2–3 days to complete and requires tissue culture facilities. The ELISA is more rapid and can be more easily standardised. A small proportion of sera from animals with no previous exposure to SVD virus will react positively in serological tests for antibody to SVD virus. The 5B7 MAb competitive ELISA (MAC-ELISA) is a reliable technique for detecting SVD antibody (1, 9) and similar results have been obtained with other ELISAs (4, 11). Results from a small proportion, 0.25%-0.45%, of sera from normal pigs are borderline or positive by the MAC-ELISA and should be retested by the VN test. Up to approximately 50% of these sera will also be positive by the VN test (i.e. 0.1–0.2% of the original population). Animals positive by ELISA, but negative by VN test can be regarded as uninfected. Repeat samples should be collected from animals positive in both tests and from cohorts. A constant or declining titre in the positive animal and the absence of antibody to SVD virus in cohorts confirms the status of the positive animal as a 'singleton reactor'. The factors responsible for 'singleton reactors' are unknown. Serological cross-reactivity with SVD virus might arise due to infection with another, as yet unidentified, picornavirus or may be due to other non-specific factors present in the serum. Identification of the isotype of antibody present in positive sera (1) can be helpful as sera from infected pigs usually contain specific IgG alone or both IgG and IgM, whereas sera from 'singleton' reactors usually contain exclusively IgM and do not convert to IgG (6). IgM/IgG isotype-specific ELISAs are also helpful in assessing the time of infection in the pig or on the infected premises. The presence of IgM, alone or together with IgG, is evidence of recent infection and indicative of virus shedding, while detection of IgG alone suggests an older exposure to infection (1).

a) Virus neutralisation (the prescribed test for international trade)

The quantitative VN microtest for antibody to SVD virus is performed using IB-RS-2 cells (or suitable susceptible porcine cells) in flat-bottomed tissue-culture grade microtitre plates.

Virus is grown on IB-RS-2 cell monolayers and stored at -20°C after the addition of an equal volume of glycerol. SVD virus has been found to be stable under these conditions for at least 1 year. The sera are inactivated at 56°C for 30 minutes before testing. A suitable medium is Eagle's complete medium/LYH with antibiotics.

The test is an equal volume test in 50 μl volumes:

- i) Starting from a 1/4 dilution, sera are diluted in a twofold dilution series across the plate, two rows of wells per serum.
- ii) Previously titrated virus is added; each 50 μl unit volume of virus suspension contains about 100 TCID₅₀ (50% tissue culture infective dose).
- iii) Controls include at least a weak positive serum and a negative serum, a cell control, a medium control and a virus titration used to calculate the actual virus titre used in the test.
- iv) Incubate at 37°C for 1 hour with the plates covered.
- v) A cell suspension at 10^6 cells/ml is prepared in medium containing 10% bovine serum for cell growth. 50 μl of cell suspension is added to each well.
- vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 5% carbon dioxide at 37°C for 2–3 days.
- vii) Microscopic readings are feasible after 48–72 hours; the plates may be finally fixed and stained routinely on the third day. Fixation is effected with 10% formalin/saline for 30 minutes; staining is done by immersion in 0.05% methylene blue in 10% formalin for 30 minutes. The plates are rinsed in tapwater.
- viii) Positive results are blue-stained cell sheets; the negative wells are empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture at the 50% end-point. The test is considered to be valid when the amount of virus actually used per well is between $10^{1.5}$ and $10^{2.5}$ TCID₅₀, and the positive standard sera are within twofold of their expected titre.
- ix) *Interpretation of the results:* At the Pirbright, OIE Reference Laboratory for SVD (see footnote 1), VN titres less than or equal to 1/11 are considered to be negative. Titres of 1/16 to 1/32 are doubtful and VN titres of 1/45 or more are regarded as positive. However, as titres depend on the cell system used, laboratories should establish their own criteria by reference to standard reagents available from the OIE Reference Laboratory.

b) Enzyme-linked immunosorbent assay

In the ELISA developed by Brocchi *et al.* (1), the SVD viral antigen is trapped to the solid phase using the MAb 5B7. The ability of test sera to inhibit the binding of peroxidase-conjugated MAb 5B7 to the trapped antigen is then evaluated. Finally, the amount of conjugated MAb bound is detected by the addition of substrate and chromogen.

- i) ELISA plates are coated with 50 μl /well of MAb 5B7 at a saturating dilution in carbonate/bicarbonate buffer, pH 9.6, by overnight incubation at 4°C .
- ii) The plates are washed three times with PBS containing 0.05% Tween 20, and 50 μl of SVD antigen (SVD virus grown in IB-RS-2 cells, clarified, filtered and inactivated) at a predetermined optimal dilution, is added to each well. The optimal dilution of antigen is determined by checkerboard titrations of antigen and conjugated MAb that define the working dilution giving an absorbance on the upper part of the linear region of the antigen titration curve (between 1.5 and 2.0 optical density units). Plates are then incubated for 1 hour at 37°C .
- iii) After three additional washes, 50 μl of diluted test sera (not inactivated) and control sera are incubated with the trapped antigen for 1 hour at 37°C . Three-fold dilution series of sera are obtained directly in ELISA wells by adding 10 μl of serum to 65 μl of buffer (1/7.5 dilution) then transferring 25 μl to sequential wells containing 50 μl of buffer, mixing, and finally discarding 25 μl .
- iv) After incubation for 1 hour, 25 μl of an optimal dilution of peroxidase-conjugated MAb 5B7 (see step ii above) is added to each well and the plates are incubated at 37°C for a further 1 hour.
- v) After a final series of washes, the colorimetric reaction is developed by distributing 50 μl per well of the substrate solution (for example 0.5 mg/ml orthophenylene-diamine in phosphate/citrate buffer, pH 5, containing 0.02% H_2O_2).

- vi) The reaction is stopped after 10 minutes by adding 50 µl of 2 N H₂SO₄. The absorbance is read at the appropriate wavelength using a microplate reader.

Antigen, sera and conjugate are diluted in PBS, pH 7.4, containing 0.05% Tween 20 and 1% yeast extract; the dilution buffer for sera contains, in addition, 1.0% mouse serum to prevent nonspecific binding of pig serum to MAb 5B7 either coated to the plate or conjugated to peroxidase.

- vii) *Controls*: Four wells on each plate containing all reactants except test serum confirm the maximum absorbance reading for the antigen; convalescent pig serum at four selected dilutions; negative pig serum; a low positive standard pig serum.
- viii) *Interpretation of the results*: Reactions are expressed as the percentage inhibition by each test serum of the MAb reaction with the SVD antigen. Sera are considered to be positive when producing an inhibition ≥80% at the 1/7.5 dilution; negative when producing an inhibition <70% at the 1/7.5 dilution; doubtful when producing an inhibition ≥70% and <80% at the 1/7.5 dilution. The second dilution (1/22.5) provides an indication of the level of antibodies: strongly positive sera show >80% inhibition at both 1/7.5 and 1/22.5 dilutions, while Sera registering >80% inhibition at the 1/7.5 dilution but <50% inhibition at the 1/22.5 dilution are considered to be low positive or borderline. All positive, borderline and doubtful sera should be confirmed using the VN test.

STANDARD REFERENCE SERA FOR SVD SEROLOGY

The OIE Reference Laboratory, Pirbright, UK maintains a panel of reference sera that have been extensively validated by the National SVD Reference Laboratories of the Member States of the European Union.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No commercial SVD vaccines are currently available. Standard sera can be obtained from the OIE/FAO WRL for FMD (see footnote 1). MAb 5B7 is available from the OIE Reference Laboratory for swine vesicular disease in Italy (see Table given in Part 3 of this *Terrestrial Manual*).

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NB: There are OIE Reference Laboratories for Swine vesicular disease (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).