

AUSTRALIAN  
STANDARD  
DIAGNOSTIC  
TECHNIQUES  
FOR ANIMAL  
DISEASES

STANDING  
COMMITTEE ON  
AGRICULTURE  
AND RESOURCE  
MANAGEMENT

ANIMAL HEALTH  
COMMITTEE

SUB-COMMITTEE ON  
ANIMAL HEALTH  
LABORATORY  
STANDARDS

# Porcine Parvovirus Infection

*Virology and Serology*

*G. W. Burgess*

Department of Biomedical and Tropical Veterinary Science,  
James Cook University of North Queensland, Townsville,  
Qld 4811, Australia.

---

## Contents

1. Introduction	3
2. The disease	3
2.1. Individual infection	3
2.2. Passive immunity	3
2.3. Epidemiology	3
2.4. Manifestations of infection	3
2.5. Vaccination	4
2.6. Laboratory diagnosis of porcine parvovirus reproductive failure	4
3. Antigen demonstration	4
3.1. Haemagglutination	4
3.2. Enzyme-linked immunosorbent assay for antigen detection	4
3.3. Immunodiffusion for antigen detection	4
4. Antibody demonstration	5
4.1. Haemagglutination-inhibition procedure	5
4.2. Recently formed antibody	5
4.3. Production of viral antigen	6
4.4. Enzyme-linked immunosorbent assay for antibody determination	6
4.5. Immunodiffusion for antibody detection	6
5. Isolation of virus	6
5.1. Glycine extraction technique	7
5.2. Immunoperoxidase staining of cell cultures	7
6. Histopathology	7
7. References	7
8. Appendixes	8
8.1. Appendix 1 — Reagents	8

**Porcine Parvovirus Infection**

First published as: *Porcine Parvovirus Reproductive Failure* by R. H. Johnson and H. S. Joo, published by the Australian Bureau of Animal Health (1978).

**2**

Revised by R. T. Jones and published by the Australian Bureau of Animal Health (1980).

## 1. Introduction

Between 85% and 95% of Australian pig herds have been shown to be endemically infected with porcine parvovirus (PPV). Once introduced to a farm, the extreme resistance of this virus to environmental decay ensures that the farm may be perpetually infected. Fortunately, there is only one antigen type of PPV, and active infection is a 'once-only' phenomenon, as the virus usually induces very high levels of immunity persisting for the life of the pig. Active infection with PPV causes no clinical signs in neonates or adults, and such active infection is only of proved consequence when it coincides with a pregnancy status of fewer than 55 days. Under these conditions, transplacental infection of the conceptus may associate with reproductive failure in the form of damaged litters.

The major effect of porcine parvovirus infection is the reduction in litter size as a result of infection of embryos and foetuses. This is predominantly seen as return to service, small litter size or mummified piglets. The history of PPV infection in a herd can easily be ascertained by carrying out a serological survey which includes animals from all parity groups. Affected foetuses will contain PPV, specific antibody or both. Active infection can also be monitored using serial bleeding of high risk groups such as the gilts. Vaccination appears to be effective in controlling the losses from this disease. Spontaneous recovery from PPV infection in small to medium sized herds may be more common than we had previously anticipated.

## 2. The Disease

### 2.1. Individual Infection

Active infection with viraemia can only occur in pigs which have no detectable passive or active immunity as measured by standardised haemagglutination-inhibition (HI) procedures. Infection occurs by the oral route in nature, usually from ingestion of faeces containing virus. In an actively infected pig, a viraemia occurs between one and six days post infection (p.i.), persisting for one to five days, coincident with an irregular panleucopenia. Virus is excreted principally in faeces for up to 14 days p.i. and antibody is detected in HI seven to nine days p.i. reaching titres up to 15 000 within 21 days. This high titre may diminish slowly over the lifetime of the pig. Actively immune pigs in general have antibody titres greater than 256.

### 2.2. Passive Immunity

Passive immunity persists for between four and nine months. The duration of passive immunity is directly linked with the dams titre. This implies that litters from recently infected dams (usually gilts) will receive high levels of passive

immunity. As mating usually occurs at six to seven months of age, it follows that many gilts (up to 50%) will not have active immunity at the time of service, and may become actively infected when pregnant. Haemagglutination-inhibition titres over 256 in serum from pigs older than four months imply active immunity.

### 2.3. Epidemiology

Virus is usually introduced into PPV-free farms by purchase of infected pigs. The spread of virus through the farm depends on the type of husbandry practised. On farms where pigs are penned in groups reproductive failure will become evident about two months after the introduction of virus and will continue for a further two to three months, by which time up to 100% of pigs will usually have become infected. Thereafter most animals over one year of age will have developed immunity, and susceptible stock will localise to gilts losing passive immunity.

Where pigs are individually penned, build-up of virus after introduction is minimised and spread is irregular. Occasional reproductive failure from two months after the introduction of virus will become evident. Infection may persist for years, in both gilt and older sow groups. Susceptible pigs may be found in all age groups. Some of these herds may revert to uninfected status.

### 2.4. Manifestations of Infection

Viraemia in a sow or boar, at the time of service, may result in return to service or the production of small litters. This is believed to be associated with the presence of virus in semen or ovarian fluids, this free virus adversely affecting conceptus, particularly in antibody-free dams. Infection of the sow at 1-35 days gestation may result in foetal death with resorption. The pregnancy will be maintained if more than four foetuses survive. There is a suggestion, however, that infection at this stage of gestation may rarely result in persistently infected apparently immunologically tolerant animals.

Infection of sows at a gestational age of less than 55 days associates with conceptus infection and damage 7-30 days later. Various degrees of damage to the full term litter may result. The earlier that the dam is infected, the greater the overall damage to the litter. As each foetus has individual placentation, infection is not synchronised and there is an individual response to infection in each member. This results in various combinations of advanced mummification (death about 50-65 days gestation), partial mummification (death between 65 and 100 days gestation), stillbirth, early neonatal death or survival. Occasionally, damaged litters may be aborted. If there are no conceptus survivors, the pregnancy may continue past full term.

Where the sow is infected at a gestational stage of 55–80 days, virus reaches the foetuses after they have become relatively immunocompetent and obvious damage to the litter members is rare. The majority are born with active immunity and residual virus in tissues. Dam infection after 80 days gestation is not likely to allow time for the virus to cross to the foetuses, and infection is rare.

The relatively frequent occurrence of PPV or PPV-antibody in normal newborn piglets may on isolated occasions lead to misdiagnosis where the piglets concerned are pathologically abnormal due to other coincident factors.

### 2.5. Vaccination

Vaccination strategies aim to produce a level of immunity in pregnant sows sufficient to reduce the viraemia to a level where virus is unlikely to cross the placenta. A range of vaccines is available overseas and to date one vaccine has been introduced in Australia. Most vaccines contain inactivated virus with an adjuvant suitable for intramuscular inoculation. In general the serological responses following vaccination are lower than those which follow natural infection. Titres will decline with time.

The protective immunity appears to relate to immune memory at least as much as it does to circulating antibody titres. Some animals with titres undetectable by HI may be protected. Caution must be exercised when interpreting titres of serum samples collected from vaccinated animals.

### 2.6. Laboratory Diagnosis of Porcine Parvovirus Reproductive Failure

Diagnosis of PPV infection is based on the demonstration of PPV or PPV antigen in mummified foetuses less than 14 cm crown-rump length (CRL), or of PPV antibody in foetuses greater than 16 cm CRL. Foetuses between 14 and 16 cm CRL should be avoided as they may give indeterminate results, possibly due to antigen-antibody complexes.

## 3. Antigen Demonstration

Viral antigen in foetuses less than 14 cm CRL, may be demonstrated by immunofluorescence, immunodiffusion, haemagglutination (HA) or enzyme-linked immunosorbent assay (ELISA).

### 3.1. Haemagglutination

- (a) Grind about 5 g of any visceral tissues (intestines, kidneys, liver, lungs).
- (b) Suspend in about five times the volume of phosphate buffered saline (PBS, see 8.1.1.).
- (c) Freeze and thaw three times (alternatively ultrasonicate for five minutes), and clear by centrifugation at 2000 g for 10 min.

- (d) Test supernatant for HA using 0.6% guinea-pig erythrocytes at room temperature (25–28°C) over a dilution range of 2–1024 in PBS diluent. Read after two hours.
- (e) Check samples which react in HA for specificity by HI (see 4.1.) using known high titre and known non-reactor PPV reference serum.

### 3.2. Enzyme-linked Immunosorbent Assay for Antigen Detection

The ELISA for antigen detection developed at James Cook University of North Queensland captures the antigen from the sample using a polyclonal antibody adsorbed to plastic wells. The presence of viral antigen is indicated by a monoclonal antibody conjugated to an enzyme. The colour produced is related to the antigen concentration in the sample. The method is more sensitive than HA.

The assay should be carried out according to the manufacturer's instructions. Single dilutions of antigen are used in the test. The optical density of the test samples is compared with a standard curve and the titres are calculated from the values allocated to the standard curve samples.

### 3.3. Immunodiffusion for Antigen Detection

An immunodiffusion technique has been described for both antigen and antibody detection (Too *et al.*, 1983). The method is sufficiently sensitive to detect viral antigen in mummified foetuses. Tissues can be prepared as in 3.1. with the tissues being homogenised in borate buffer (pH 8.6).

- (a) Prepare the gel by suspending 1% w/v agarose (Seakem Agarose, FMC Bioproducts, available in Australia from Edwards Laboratories, PO Box 60, Narellan, NSW 2567. Tel. (046) 471 444; Fax (046) 552 644) in borate buffer (pH 8.6) containing 0.9% w/v boric acid ( $H_3BO_3$ , 0.145 mol/L), 0.2% w/v sodium hydroxide (NaOH, 0.05 mol/L) and 0.01% sodium azide ( $NaN_3$ , 0.0015 mol/L). After dissolving the gel and cooling to 56°C, aliquots of 15 mL are dispensed into 90 mm plastic petri dishes and allowed to cool.
- (b) Seven well patterns are cut in the gel so that the 6 mm tubes are positioned 2.5 mm apart. Up to six such patterns can fit onto one plate.
- (c) The method is standardised using control serum from infected pigs and antigen prepared as in 4.3. A straight line is produced midway between the antibody and antigen wells. The well nearest the side is numbered 1 and the samples are added in a clockwise direction.
- (d) For antigen detection, 50 µL of control serum can be loaded into the central well and wells 1, 3 and 5 loaded with control antigen. Test samples are loaded into wells 2, 4 and 6.

- (e) Plates are incubated at 18–22°C for 48 hours.
- (f) A small amount of antigen may be detected by a bending of the control lines.

Large amounts of antigen can produce a line which is continuous with the reference lines.

#### 4. Antibody Demonstration

Antibody may be demonstrated by serum neutralisation, immunodiffusion ELISA or HI. The ELISA may also be carried out on antibody eluted from filter paper. There is a close correlation with serum antibody titres.

Serum can be readily obtained from heart blood of stillborn or large foetuses. From partially mummified foetuses, serum-equivalent may be obtained from fluid in body cavities, or, alternatively, viscera with the stomach removed may be placed in a plastic bag overnight at 4°C, exudate collected and cleared by centrifugation at 2000 g for 10 min.

##### 4.1. Haemagglutination-inhibition Procedure

- (a) To the first well of rows A and B of a microtitre plate, pipette 25 µL of serum heat-inactivated at 56°C for 30 min. Add 75 µL of a 25% solution of acid washed kaolin in borate saline at pH 9 (see 8.1.2.). Shake thoroughly and incubate at room temperature for 20 min. Deposit the kaolin by centrifugation at 1000 g for 20 min. This procedure removes non-specific inhibitors of haemagglutination.
- (b) Without removing deposited kaolin, add 25 µL of 15–25% washed guinea-pig erythrocytes to each of the wells. Mix gently and incubate at room temperature for one hour. Centrifuge for 10 min at 1000 g. This removes non-specific agglutinins to guinea-pig erythrocytes. The supernatant fluid is regarded as a 1:4 dilution of the original sample.
- (c) Add 50 µL of PBS to each of the remaining wells on the plate. Transfer 50 µL of the treated serum from the first well to the second well in each of the rows A and B. Using 50 µL aliquots, carry out serial dilutions of the samples to the end of the plate. This gives a dilution range of 1:8 to 1:8192.
- (d) To the control row B add 50 µL of PBS to replace the virus. To each of the wells in row A add 50 µL of viral suspension containing four haemagglutinating units (HAU) of virus. Incubate at room temperature for one hour.
- (e) Add 50 µL of a 0.6% suspension of washed guinea-pig erythrocytes (see 8.1.3.) to each well and allow to settle for one hour at room temperature.
- (f) The end point of the titration is taken as the last well to show complete inhibition of haemagglutination. The result is expressed as a reciprocal of this serum dilution.

The procedure described above is repeated for each of the serum samples to be tested. The test is facilitated if it is carried out in microtitre plates and these centrifuged in a plate carrier in a laboratory centrifuge. If this equipment is not available the adsorption steps will have to be carried out in tubes. Pipetting and transfer of reagents is done much more quickly if a multi-channel pipette is used.

Each batch of tests should include a range of serum control of known titre. Controls for the erythrocyte suspension and a titration of the haemagglutinin must be used. The titre of the control sera should not vary more than one well from the established titres. The haemagglutination titration should show complete haemagglutination at a 1:4 dilution.

The following practical points are made.

- (a) Kaolin may remove specific antibody from sera which have been diluted more than 1:4 before treatment.
- (b) Even with kaolin treatment, haemagglutination in the very low dilutions of serum may require careful interpretation due to the frequent occurrence of 'slip' where agglutinated red cells tend to form a button, similar to absence of haemagglutination. Such slip agglutination forms a red cell button slightly larger and rougher than the controls in wells 2 and 8. Considerable care must be taken, therefore, when assessing low levels of immunity.
- (c) Human erythrocytes are of slightly lower sensitivity to PPV HA than are guinea-pig erythrocytes, but give equivalent results from HI. Human erythrocytes have the advantage of long storage properties at 4°C in Alsever's solution (see 8.1.8.), and may be readily available in large volumes from blood banks (outdated stock). Care must be taken to avoid infection of staff with human pathogens.
- (d) Antigen produced in the presence of foetal bovine serum which contains inhibitors (see 4.3.) or antigen fluids containing residual cell debris, may lose titre if left to stand at room temperature, because of clumping or adsorption of virus. It is always wise, therefore, to check that diluted antigen does in fact contain four HAU before adding to serum dilutions. Frequently, antigen which loses HA titre on storage can be reactivated by ultrasonication for five minutes, followed by filtration through a 450 nm millipore to remove fine cell debris. Stock antigen is best stored at 4°C, as frozen antigen needs at least six hours to stabilise after thawing.

##### 4.2. Recently Formed Antibody

For epidemiological studies, and retrospective diagnosis of PPV reproductive failure where no foetal material is available, tests have been devised to measure the amount of IgM and to

relate this to the possible time of infection. The tests used are usually based on a mercaptoethanol reduction technique.

#### 4.3. Production of Viral Antigen

Viral antigen may be produced in cell culture, or obtained from infected mummified foetuses as in 3. For production in cell culture, primary or low passage porcine monolayers may be used. The swine testis (ST) cell line is considered to one of the best continuous cell lines for producing stock viral antigen.

The following practical points are made:

- (a) A major problem in production is the apparent presence of non-specific inhibitors in many bovine serum supplements used. Adult sera are least prone to cause such inhibition, and sera for supplementation may be selected by HI (omitting kaolin) and selecting sera which give lowest titres (<16). To further eliminate the effect of such inhibitors, if cells are infected in suspension it is suggested that serum be omitted during the adsorption period. Serum is then added to 5% concentration, and infected monolayers are incubated at 37°C for 24–48 hours to the stage where small islands of cells appear. Media is then changed to a 1% serum supplement.

If cells are infected as monolayers, the virus should be adsorbed for one hour at 37°C to washed half-formed monolayers, and a maintenance medium with 1% serum supplement then added.

- (b) The time of harvest will depend on the viral challenge dose used. As obvious unstained cytopathic effect is irregular, the optimal method for determining peak yields is to monitor the concentration of antigen in the supernatant using HA or ELISA.

At the stage of maximal titre, the monolayer is frozen and thawed three times. The yield is centrifuged at 5000 g for 10 min to clear, adjusted to slightly alkaline pH with sodium hydroxide (NaOH), and stocks are made in aliquots stored at 4°C. If antigen is stored at -20°C, thawing of stock requires at least six hours at room temperature for HA to stabilise.

#### 4.4. Enzyme-linked Immunosorbent Assay for Antibody Determination

The ELISA developed at James Cook University of North Queensland is based on competition between the porcine antibody and a monoclonal antibody conjugated to an enzyme. The colour produced is reduced when the sample contains antibody. The sensitivity is greater than that observed with HI and samples may be collected as dried blood absorbed to filter paper.

The increased sensitivity can be an advantage when low maternal antibody titres and

some vaccine responses are being monitored. However, the responses following natural infection are such that the added sensitivity is of little advantage. The advantage of the ELISA lies in the convenience of the sampling and the laboratory technique. The results are sufficiently distinct that they can be evaluated without a plate reader.

Agreements for the marketing of this assay have been completed and it is anticipated that the product will be launched as a self contained kit by the Australian diagnostics manufacturer TropBio before the end of 1993 [James Cook University Tropical Biotechnology Pty Ltd, James Cook University, Townsville, Qld 4811. Tel. (03) 077 814 328; Fax (077) 791 526].

The assay should be carried out according to the manufacturer's instructions. Single dilutions of serum are used in the test. The optical density of the test samples is compared with a standard curve and the titres are calculated from the values allocated to the standard curve samples.

#### Immunodiffusion for Antibody Detection

Immunodiffusion technique has been described for both antigen and antibody detection (Too *et al.*, 1983). The method correlates well with HI for antibody detection. While this is a relatively insensitive test, the titres produced following natural infection are high and readily detected using this assay. No published results on the use of this test for measuring vaccine titres is available at this stage. The method is sufficiently sensitive to detect viral antigen in mummified foetuses.

- (a) Immunodiffusion plates are prepared (see 3.2.).
- (b) Control antigen is prepared as in 2.2 and 50 µL aliquots are placed in the central wells. Reference antisera are placed in wells 1, 3 and 5.
- (c) Test samples are loaded into wells 2, 4 and 6.
- (d) With a negative result, the control lines extend between the control antibody and antigen wells without deviation.

Low titre samples cause a deviation in the ends of the control lines.

High titre samples may have antibody excess and a broad diffuse band close to the antigen well is seen. In this case it is advisable to dilute the serum and repeat the test.

### 5. Isolation of Virus

Diagnosis of PPV in mummified foetuses (14 cm CRL) can be made by viral isolation, immunofluorescence, haemagglutination, immunoelectronmicroscopy or ELISA. For viral isolation, higher yields of viable virus will be obtained from non-mummified members of a damaged litter. Spleen or kidney extracts from stillborn members of a litter are adsorbed to partly

formed monolayers of susceptible cells, using procedures as described for antigen production. If there are surviving members of a damaged litter, the kidneys should be autocultured.

Appearance of free antigen coincides with development of nuclear inclusions or specific antigen in stained coverslips. Cell cultures may be stained using immunoperoxidase to demonstrate the presence of viral antigens.

An excellent method for improving HA yields from test monolayers is to use a glycine extraction technique which frees cell-associated virus haemagglutinin (see 8.1.4.).

It should be noted that in autoculture kidneys from infected litter members virus may appear within three to seven days, while samples adsorbed to indicator monolayers may take up to 21 days to develop inclusions and HA. In such cases, it is advisable to passage the autocultured kidneys even when antigen can not be demonstrated by HA in the supernatant.

#### 5.1. Glycine Extraction Technique

- (a) Decant media.
- (b) Wash monolayer rapidly with glycine buffer (see 8.1.4.).
- (c) Cover monolayer with a minimal volume of glycine buffer and hold at room temperature for one hour.
- (d) Remove glycine buffer centrifuge at 2000 g for 10 min and test supernatant fluids for HA.
- (e) Media may be renewed as monolayers are still viable after glycine treatment.

#### 5.2. Immunoperoxidase Staining of Cell Cultures

Viral antigen in cell cultures may be demonstrated using immunoperoxidase staining. The method is more sensitive than staining for inclusion bodies. The specificity can be improved by using monoclonal antibodies which react with PPV. The method described is for an indirect procedure using an alcohol soluble coloured product. Permanent mounts can be produced using an aqueous mounting medium or by changing the substrate.

All steps are carried out at room temperature.

- (a) Monolayers are fixed with buffered formalin acetone for one minute.
- (b) Wash. (All washing steps are carried out three times in PBS).
- (c) Dried coverslips placed in a humid chamber with cells facing up are covered with antisera to PPV. The chambers are placed at 37°C for one hour and then washed.
- (d) Antiglobulin conjugated to horseradish peroxidase is added to coverslips in the same manner as (b).
- (e) Wash.
- (f) Cells are immersed in 3-amino-9-ethylcarbazole substrate for 15 min, then washed in tap water and mounted in buffered glycerol.

The cells are examined under a light microscope. Counterstains such as haematoxylin can be used.

## 6. Histopathology

Histological lesions are seen in foetuses which have become infected after about 70 days of gestation (Forman *et al.*, 1977; Hogg *et al.*, 1977). A non-suppurative inflammation occurs in the central nervous system, eye, liver, kidney, myocardium, endocardium, lung, gastrointestinal tract, mesentery, skeletal muscle and foetal membranes. In the brain and meninges, perivascular cuffing with mononuclear cells including lymphocytes and plasma cells is a consistent lesion. Suppurative bronchopneumonia may also be present.

The lesions are not pathognomonic. However, their presence suggests the diagnostic possibility of PPV infection and can be a valuable aid where specimens are not available for demonstration of virus, antigen or specific antibody.

## 7. References

- Runnels, L.J., Turek, J.J., and Gustafson, D.P. (1982). Observations on pseudorabies virus, porcine parvovirus, and porcine enterovirus in porcine embryo transfer. *Proceedings of the United States Animal Health Association* 86, 391-4.
- Burgess, G.W., Heytman, M.J., and Lee, R.P. (1987). Advances in the techniques for the monitoring of porcine parvovirus infection. In 'Advances in Veterinary Science'. (Ed. P. M. Outteridge.) pp. 60-1. (Australian Veterinary Association: Sydney.)
- Cutler, R.S., Molitor, T.W., Leman, A.D., and Werdin, R.E. (1983). Farm studies of porcine parvovirus infection. *Journal of the American Veterinary Medical Association* 182, 592-4.
- Cutlip, R.C., and Mengeling, W.L. (1975). Pathogenesis of in utero infection. Experimental infection of eight and ten week-old porcine fetuses with porcine parvovirus. *American Journal of Veterinary Research* 36, 1751-4.
- Forman, A.J., Lenghaus, C., Hogg, G.G., and Hale, C.J. (1977). Association of a parvovirus with an outbreak of foetal death and mummification in pigs. *Australian Veterinary Journal* 53, 326-9.
- Hogg, G.G., Lenghaus, C., and Forman, A.J. (1977). Experimental porcine parvovirus infection of foetal pigs resulting in abortion, histological lesions and antibody formation. *Comparative Pathology* 87, 539-49.
- Joo, H.S., Donaldson-Wood, C.R., and Johnson, R.H. (1975). A microneutralization test for the assay of porcine parvovirus antibody. *Archives of Virology* 47, 337-41.
- Joo, H.S., Donaldson-Wood, C.R., and Johnson, R.H. (1976a). A standardised haemagglutination inhibition test for porcine parvovirus antibody. *Australian Veterinary Journal* 52, 422-4.
- Joo, H.S., Donaldson-Wood, C.R., and Johnson, R.H. (1976b). Rapid diagnostic techniques for detection of porcine parvovirus infection in mummified foetuses. *Australian Veterinary Journal* 52, 51-2.
- Joo, H.S., and Johnson, R.H. (1976). Porcine parvovirus. A review. *Veterinary Bulletin* 46, 653-60.
- van Leengoed, L.A., Vos, J., Gruys, E., Rondhuis, P., and Brand, A. (1983). Porcine parvovirus infection. Review and diagnosis in a sow herd with reproductive failure. *Veterinary Quarterly* 5, 131-41.

- Leslie-Stein, P., and Kirkbride, C. (1983). Comparison of diagnostic methods for *in utero* parvovirus infection of swine fetuses. In 'Proceedings of the Third International Symposium of the World Association of Veterinary Laboratory Diagnosticians'. pp. 111-17. (Ames, Iowa, USA.)
- Mengeling, W.L. (1986). Porcine parvovirus infection. In 'Diseases of Swine'. 6th edn. (Ed. A.D. Leman.) (Iowa State Press: Ames, Iowa, USA.)
- Mengeling, W.L., Paul, P.S., and Brown, T.T. (1980). Transplacental infection and embryonic death following maternal exposure to porcine parvovirus near the time of conception. *Archives of Virology* 65, 55-62.
- Morrison, R.B., and Joo, H.S. (1984). Acute reproductive losses due to porcine parvovirus infection in a swine herd. Herd observations and economic analysis of the losses. *Preventive Veterinary Medicine* 2, 699-706.
- Paul, P.S., Mengeling, W.L., and Pirtle, E.C. (1982). Duration and biological half-life of passively acquired colostral antibodies to porcine parvovirus. *American Journal of Veterinary Research* 43, 1376-9.
- Pointon, A.M., Surman, P.G., McCloud, P.I., and Whyte, P.B.D. (1983). The pattern of endemic parvovirus infection in four pig herds. *Australian Veterinary Journal* 60, 166-71.
- Sorensen, K.J., and Askaa, J. (1981). Fetal infection with porcine parvovirus in herds with reproductive failure. *Acta Veterinaria Scandinavica* 22, 162-70.
- Too, H.L., and Love, R.J. (1986). Some epidemiological features and effects on reproductive performance of endemic porcine parvovirus infection. *Australian Veterinary Journal* 63, 50-3.
- Too H.L., Seaman, J.T., Littlejohns I.R., and Love R.J. (1983). Evaluation of a gel diffusion precipitin test for porcine parvovirus. *Australian Veterinary Journal* 60, 161-5.
- Vannier, P., Tillon, J.P., Cariolet, R., and Madec, F. (1984). A seroepizootiological study of parvovirus in pig herds. *Zentralblatt für Veterinärmedizin Reihe B*, 31, 36-45.
- Wrathall, A.E., Wells, D.E., Cartwright, S.F., and Frerichs, G.N. (1984). An inactivated, oil-emulsion vaccine for the prevention of porcine parvo-induced reproductive failure. *Research in Veterinary Science* 36, 136-43.
- Wrathall, A.E., and Mengeling, W.L. (1979a). Effect of porcine parvovirus on the development of fertilized pig eggs *in vitro*. *British Veterinary Journal* 135, 249-54.
- Wrathall, A.E., and Mengeling, W.L. (1979b). Effect of transferring parvovirus-infected fertilized pig eggs into seronegative gilts. *British Veterinary Journal* 135, 255-61.
- Wrathall, A. E., and Mengeling, W.L. (1979c). Effect of inseminating seropositive gilts with semen containing porcine parvovirus. *British Veterinary Journal* 135, 420-5.

## 8. Appendixes

### 8.1. Appendix 1 — Reagents

#### 8.1.1. Phosphate Buffered Saline (PBS)

- (a) Solution A
- |   |       |
|---|-------|
| NaCl  | 8.0 g |
| Potassium chloride, KCl   | 0.2 g |
| Potassium dihydrogen phosphate, KH <sub>2</sub> PO <sub>4</sub>           | 0.2 g |
| Disodium hydrogen phosphate, Na <sub>2</sub> HPO <sub>4</sub> (anhydrous) | 0.5 g |
- Dissolve in 800 mL distilled water.
- (b) Solution B
- |  |       |
|--|-------|
| Magnesium chloride, MgCl <sub>2</sub> ·6H <sub>2</sub> O | 0.1 g |
| CaCl <sub>2</sub> ·6H <sub>2</sub> O                     | 0.1 g |
- Dissolve in 100 mL distilled water

Add Solution A to Solution B and make up to 1 L with distilled water. If required sterile, filter through a 220 nm millipore or Seitz filter. Alternatively, autoclave Solutions A and B separately, allow to cool and combine under sterile conditions. The pH of the final solution should be 7.1-7.2.

#### 8.1.2. Acid-washed kaolin (pH 9)

- (a) Solution A
- |                         |         |
|-------------------------|---------|
| NaCl                    | 87.68 g |
| Distilled water to 1 L. |         |
- (b) Solution B
- |                                |        |
|--------------------------------|--------|
| H <sub>3</sub> BO <sub>3</sub> | 30.9 g |
| Hot distilled water to 1 L.    |        |
- (c) Borate saline pH 9
- |                             |        |
|-----------------------------|--------|
| Solution A                  | 80 mL  |
| Solution B                  | 100 mL |
| NaOH, 1 mol/L               | 24 mL  |
| Hot distilled water to 1 L. |        |
- Check that the pH is 9.
- (d) Acid-washed kaolin/borate saline
- |                          |  |
|--------------------------|--|
| 25 g acid washed kaolin  |  |
| Borate saline to 100 mL. |  |

#### 8.1.3. 0.6% Guinea-pig Erythrocytes

Wash erythrocytes three times in PBS at 1000 g for 10 min. Resuspend erythrocytes at about 10% concentration in PBS acidified to pH 6.0, and store for up to two weeks at 4°C. To make 0.6% concentration, use of the haematocrit is essential. The haematocrit reading for percentage of erythrocytes in stock solution is obtained and by use of the following formula any volume of the required concentration can be made.

$$\text{Volume of stock required} = \frac{(\% \text{ suspension required} \times \text{volume required})}{(\text{haematocrit} \% \text{ concentration of stock})}$$

For example, if 20 mL of 0.6% erythrocytes are required from a 9% stock:  $[(0.6 \times 20)/9] = 1.33 \text{ mL}$ . Add 1.33 mL of stock erythrocytes to 18.67 mL of PBS.

#### 8.1.4. Glycine Buffer

- |                            |        |
|----------------------------|--------|
| NaCl                       | 0.59 g |
| Glycine                    | 1.50 g |
| Distilled water to 100 mL. |        |
- Bring pH to 9 by adding 1-1.5 mL of 2 mol/L sodium hydroxide. Sterilise by autoclaving at 120°C for 15 min.

#### 8.1.5. Rapid Haematoxylin and Eosin Stain (Coverslips)

- (a) Wash coverslip in PBS (five seconds).
- (b) Fix in Bouin's fixative (five seconds).
- (c) Remove picric acid [(O<sub>2</sub>N)<sub>3</sub>C<sub>6</sub>H<sub>2</sub>OH] with 70% ethanol (two times 30 s).
- (d) Stain haematoxylin (four minutes).
- (e) Blue in tap water (five seconds).
- (f) Counterstain 1% eosin (10 s).
- (g) 90% Ethanol (10 s).



- (h) Absolute alcohol two times one minute (essential last alcohol is 'clear').
- (i) Xylol (30 s) — 'milky' indicates dehydration inadequate.
- (j) Mount after air drying (two minutes).

*N.B.* Coverslip racks are often toxic due to xylene contamination, and nucleoprotein of unfixed cells may coagulate. It is preferable to fix monolayers before putting into racks, or to stain in glass tubes.

#### 8.1.6. 3-Amino-9-ethylcarbazole Substrate

3-Amino-9-ethylcarbazole	10 mg
Dimethylsulfoxide (DMSO)	6 mL
Acetate buffer, 0.02 mol/L (pH 5.0–5.2)	50 mL
Hydrogen peroxide, H <sub>2</sub> O <sub>2</sub> , 0.3%	0.4 mL
Adjust the final pH to 5.5.	

#### 8.1.7. Buffered Formalin Acetone

Na <sub>2</sub> HPO <sub>4</sub>	20 mg
KH <sub>2</sub> PO <sub>4</sub>	100 mg
Dissolve salts in distilled water	30 mL
Then add 40% v/v formaldehyde, HCHO	25 mL
Add acetone, C <sub>3</sub> H <sub>6</sub> O	45 mL
Adjust pH to 7.2 with 1 mol/L sodium hydroxide.	

#### 8.1.8. Alsever's Solution

Trisodium citrate, C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> Na <sub>3</sub> ·2H <sub>2</sub> O	8.16 g
Citric acid, C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>	0.55 g
Dextrose	20.50 g
Sodium chloride	4.20 g
Dissolve in 1 L of distilled water and sterilise by autoclaving at 68 kPa for 10 min.	