

## Isotype specific ELISAs to detect antibodies against swine vesicular disease virus and their use in epidemiology

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### SUMMARY

Isotype specific ELISAs to detect antibodies against swine vesicular disease, which may help to estimate the moment of infection, were developed and validated on sera from pigs experimentally infected with four different isolates of swine vesicular disease virus. Virus specific IgM antibodies could be detected from days 3–49 and occasionally up to day 91 after infection. IgG<sub>1</sub> antibodies were first detected at day 8 and IgG<sub>2</sub> at day 11. IgA antibodies coincided with IgG<sub>1</sub> antibodies, but antibody titres varied widely. From the results obtained with the sera from the experimentally infected pigs, we calculated the day at which 50% of the pigs had become positive (D<sub>50</sub>). A D<sub>50</sub> of 5, 4, 12, 12 and 24 days was calculated, respectively, for the appearance of antibodies in the virus neutralization test, the IgM, total IgG, IgG<sub>1</sub> and IgG<sub>2</sub> ELISA. A D<sub>50</sub> of 49 days was calculated for the disappearance of IgM antibodies. The isotype specific ELISAs proved to be valuable tools to study the epidemiology of the disease.

### INTRODUCTION

Swine vesicular disease (SVD) is an infectious disease of pigs first recognized in 1966 in Italy [1]. As the clinical signs resemble those of foot-and-mouth disease, SVD is classified as an OIE list A disease. The skin is considered the main entry site of the virus [2–4]. Inoculation on that predilection site, however, even with high doses, does not always produce clinical signs in all inoculated pigs [2]. We showed previously that infection via indirect contact, which simulates the field situation, was more effective than direct inoculation of animals [4] and produced infection in all animals shortly after introduction into the contaminated environment. The epidemiology of a disease, however, cannot be studied in an experimental setting alone. Field observations together with laboratory studies are essential to understand the epidemiology. Field epidemiological data, however, are scarce, during an outbreak veterinary authorities give priority

to the removal of the infected pigs above collecting sera first. An epidemiological field study in Great Britain revealed that the main source of infection was movement of pigs (48%), partly because infected animals were transported (16%), contaminated transport vehicles were used (21%) or due to contacts at markets (11%). A second source of infection (15%) was feeding of contaminated waste food [5]. The latter was probably the route of introduction into Great Britain.

According to EU legislation (directive 92/119/EEC) the maximum incubation period, defined as the period of time likely to elapse between exposure to the agent of the disease and the onset of clinical symptoms, is 28 days. SVD control in Europe is solely based on the destruction of infected herds. In case of an outbreak, it is essential to trace contacts leading to the source of the infection, and possible to the primary outbreak. In 1992 and 1994 nine outbreaks of SVD were recorded in the Netherlands (Table 1). The sources of infection of most outbreaks were never

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Table 1. Farm characteristics and sampling procedure of the 1992 and 1994 outbreaks

Outbreak	Date of detection	Type of farm*	Herd size				Serological positive	Sampling procedure
			Sows	Boars	piglets	fatteners		
92-01	3-7-92	B	102	3	488	0	3/11	Selective
92-02	13-7-92	B	92	1	397	11	8/124	All sows, some piglets
92-03	30-7-92	F	0	0	0	352	23/72	Two pigs per pen
92-04	29-9-92	B + F	264	2	± 1000	20	157/272	All sows, and some piglets and fatteners
92-05	29-9-92	C	0	0	0	9	8/9	All pigs
92-06	27-10-92	F	0	0	0	365	87/365	All pigs
94-01	15-2-94	C	0	0	0	0	NA	Faeces, randomly
94-02	18-2-94	F	0	0	0	22	17/22	All pigs
94-03	24-2-94	C	0	0	0	0	NA	Faeces, randomly

\* B, breeding farm; F, fattening farm; C, Collection centre.

clarified. In all cases the tracing of contacts was, based on an erroneous interpretation of the EU directive, limited to those contacts that occurred 28 days prior to the onset of clinical symptoms. Based on the seroprevalence found on those farms (Table 1) it was suggested that the time of infection might have been earlier than 28 days. For this purpose we developed and evaluated five isotype specific ELISAs to detect antibodies against SVDV, and were used to estimate the time of virus introduction on all Dutch outbreak farms diagnosed in 1992 and 1994.

## MATERIALS AND METHODS

### Virus strains

A 1971 isolate from Bulgaria, BUL/2/71, a 1981 isolate from Hong Kong, HKN/11/81 and a 1992 isolate from Italy, ITL/1/92, were obtained from the European reference laboratory at Pirbright. Strain NET/1/92 was isolated in our laboratory from specimens suspected of vesicular disease from the first outbreak in 1992 (further referred to as outbreak 92-01).

### Experimental sera

Sera collected after experimental infection with four different SVDV isolates were used to validate the ELISAs. For BUL/2/71 and HKN/11/81, sera of two animals per virus strain were available, collected at regular intervals up to 155 days after infection. For strain ITL/1/92, sera of two animals collected at regular intervals up to 91 days after infection, and for strain NET/1/92, sera of five animals collected at regular intervals up to 55 days after infection were

studied. All animals were infected by intradermal injection with a high dose ( $> 10^4$  TCID<sub>50</sub> per pig) of SVDV into the bulb of the heel and housed in high security stables of the Institute.

### Field sera

Serum samples were collected on all outbreak farms diagnosed in the Netherlands in 1992 and 1994 (Table 1). In two fattening farms, 92-06 and 94-03, all pigs were sampled, and in two breeding farms, 92-02 and 92-04, all sows and boars were sampled. All available sera were tested in the isotype specific ELISAs. For some outbreaks, only the sera positive in the virus neutralization test (VNT) were available. To check the specificity of each test, 156 negative field sera collected from 16 different farms were tested in the IgM, IgG, IgG<sub>1</sub> and IgG<sub>2</sub> ELISAs.

### 1992 outbreaks

Outbreak 92-01, 92-02, and 92-03 were diagnosed by antigen detection [7] and virus isolation after clinical suspicion. Because virological and serological SVD positive pigs originating from the Netherlands were found in Italy, a serological survey was conducted. This survey revealed outbreaks 92-04 and 92-05. An additional survey of finishing pigs at slaughterhouses in the region revealed outbreak 92-06. Only one outbreak (92-05) may have been due to purchase of infected animals. The other five outbreaks had no history of intake of infected animals. The first five outbreaks were situated within 1 km from each other. Table 1 shows the type of farm, herd size, seroprevalence and the sampling procedure followed for each outbreak farm.

### 1994 outbreaks

At the export collection centre (94-01), faecal samples were taken after SVD had been diagnosed in Italy in pigs originating from this centre. SVDV was isolated from 4 of the 5 faecal samples taken. The centre had sent pigs to another export collection centre (94-03), and to a fattening farm (94-02). The latter two outbreaks were traced because of these epidemiological links. At farm 94-02, serum samples were taken from all 21 fattening pigs, of which 17 were positive. At collection centre 94-03, 15 faecal samples were randomly collected, and 9 were found positive by virus isolation. No serum samples were collected at the collection centres, because no pigs were present.

### Monoclonal antibodies

Monoclonal antibodies (MAbs) specific for porcine IgM (CVI.28.4.1), IgG (CVI.23.3.1a), IgG<sub>1</sub> (CVI.23.49.1), IgG<sub>2</sub> (CVI.34.1.1a) and IgA (CVI.27.9.1b) have been described earlier [8]. SVD virus specific MAbs, CVI.124.8 and CVI.124.11 were produced against SVD virus isolate UKG/27/72 [9]. The MAbs were partially purified from mouse ascitic fluid, or hybridoma supernatant by 50% ammonium sulphate precipitation or protein G purification. The MAbs were dialysed against phosphate buffered saline (PBS, 0.15 M, pH 7.4) before use. MAb CVI.124.8 and the IgG, IgG<sub>1</sub> and IgG<sub>2</sub> specific MAbs were conjugated with horseradish peroxidase as described by Wilson and Nakane [10].

### IgM and IgA ELISAs

ELISA plates (Costar) were coated overnight at 4 °C with a predetermined dilution of the purified IgM or IgA monoclonal antibody diluted in 0.05 M carbonate buffer (pH 9.6). After washing, the coated ELISA plates were incubated for 1 h at 37 °C with a 1/20 dilution of each serum in duplicate wells. The sera were diluted in PBS containing 0.05% tween 80 (PBST) and 5% fetal bovine serum (PBSTF). The sera from the experimentally infected animals were also serially diluted in PBSTF to determine the titre. After washing, one of the duplicate wells was incubated with a predetermined dilution of SVD viral antigen (strain UKG/27/72) diluted in PBST. The other well was filled with PBST. The bound antigen was detected after washing and incubation with a predetermined dilution of horseradish peroxidase conjugated MAb CVI.124.8 diluted in PBSTF. After

a final washing, we incubated the plate with chromogen-substrate solution (0.4 mg/ml ortho-phenylenediamine 0.015% H<sub>2</sub>O<sub>2</sub>) for 15 min at room temperature, stationary in the dark. After 15 min, the colour reaction was stopped by the addition of 100 µl 1 M H<sub>2</sub>SO<sub>4</sub> to each well.

### IgG, IgG<sub>1</sub> and IgG<sub>2</sub> ELISAs

ELISA plates were coated overnight at 4 °C with monoclonal antibody CVI.124.11 diluted at a predetermined dilution in 0.05 M carbonate buffer (pH 9.6). After washing, alternating rows of the plates were incubated for 1 h at 37 °C with a predetermined dilution of inactivated SVDV diluted in PBST or just PBST. After washing, a 1/300 dilution of serum, diluted in PBSTF, was incubated for 1 h at 37 °C in a well with SVD antigen and a well without SVD antigen. The sera from the experimentally infected animals were also serially diluted in PBSTF to determine the titre. The plates were washed and incubated for 1 h with a predetermined dilution of a monoclonal antibody directed against swine IgG, IgG<sub>1</sub> or IgG<sub>2</sub>, respectively. After a final washing 100 µl of chromogen-substrate solution was added to each well. Colour development was allowed for 15 min, stationary in the dark, and was stopped by the addition of 100 µl 1 M H<sub>2</sub>SO<sub>4</sub> per well.

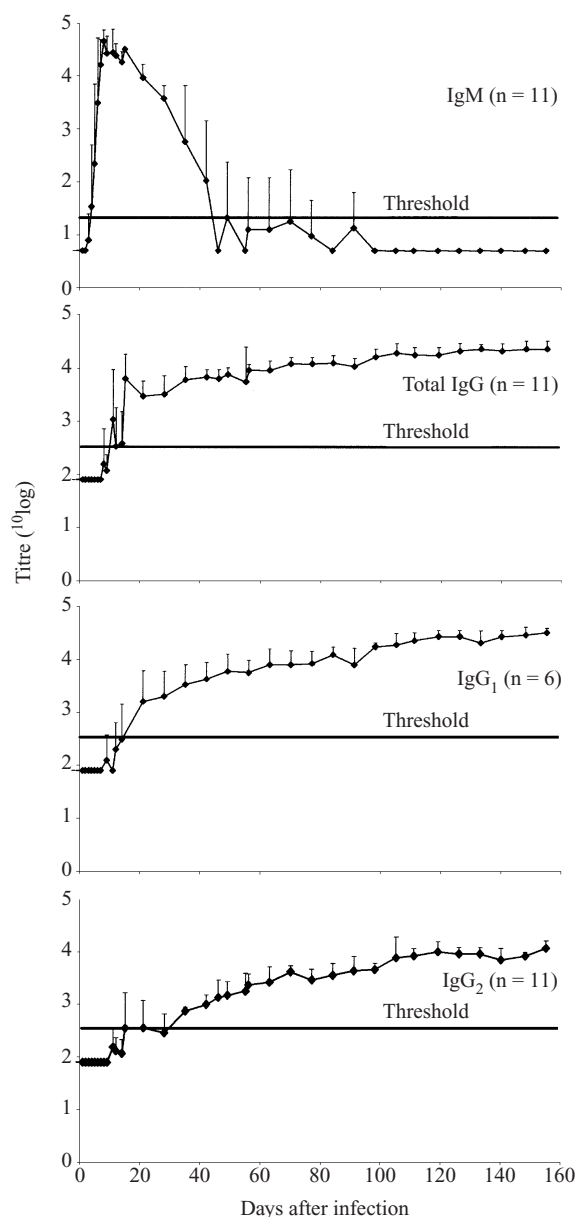
All plates were read in a microplate spectrophotometer at 492 nm. The optical density (OD) of the well without antigen was subtracted from the OD of the well with antigen. Sera with a corrected OD above 0.2 were considered positive. Titres were expressed as negative logarithm of the highest dilution in which the serum was positive. In each test, a positive control serum was titrated. A test was considered valid if the titre of the control serum was within 0.3 log of the mean titre found in previous tests. Unless indicated otherwise we made all incubations on a rotary shaker (Luckham), and washed the plates by filling and emptying them six times with tap water containing 0.05% tween 80.

### Direct liquid phase blocking ELISA

The direct liquid phase blocking ELISA was performed as previously described [11].

### Virus neutralization test

The virus neutralization test was performed as described earlier [4]. Sera with a neutralization titre



**Fig. 1.** Average IgM, IgG, IgG<sub>1</sub> and IgG<sub>2</sub> responses (bars represent the standard deviation) after experimental infection with various SVDV isolates.

equal or above the European standard serum (RS 01-04-93) were considered positive [12].

### Virus isolation

Virus isolation was performed on monolayers of IBRS-2 cells [13] as described previously [4].

## RESULTS

### Experimental infections

All inoculated pigs showed clinical signs characteristic for SVDV. The severity of the lesions, however,

**Table 2.** Start and end of responses found in the virus neutralization test, IgM and IgG ELISAs after experimental SVD infection

Test	Days after infection (based on all observations)	95% confidence interval	
		D <sub>50</sub> *	D <sub>50</sub>
<b>Start</b>			
IgM	3–7	4	3.9–5.0
VNT	4–8	5	4.9–5.9
Total IgG	8–15	12	10.3–12.9
IgG <sub>1</sub>	8–28	12	10.7–14.1
IgG <sub>2</sub>	11–35	24	20.3–28.4
<b>End</b>			
IgM	35–91	49	42.8–55.3

\* D<sub>50</sub>, The day that 50% of the animals became positive or negative (IgM) in the test.

differed between pigs receiving different strains. Strain BUL/2/71, in particular, induced less severe lesions than the other strains.

The IgM response first appeared at 3 days after infection, and at 7 days after infection all pigs were positive (Fig. 1, Table 2). Individual pigs scored positive in the IgM ELISA 0–2 days before the VNT became positive. The total IgG response started 8 days after infection, and at 15 days, all pigs were positive. The average titres and standard deviation obtained in the IgM, IgG, IgG<sub>1</sub> and IgG<sub>2</sub> ELISA are shown in Figure 1. The IgG<sub>1</sub> response was comparable to the total IgG response, starting at the same time and reaching approximately the same titres. The height of the IgG<sub>2</sub> response was lower than the total IgG response, and started approx. 2 weeks later (Fig. 1). Not all pigs became IgA positive, or stayed positive until the end of the experiment.

Using isotype specific ELISAs, different stages of infection could be identified (Table 2). Between 3 and 6 days post infection (d.p.i.), the first period was identified in which only the IgM ELISA scored positive. In individual pigs, however, this period lasted maximally 2 days, but could also be absent. This first period was followed by a second period, between 4 and 15 d.p.i., in which the pigs were positive by the VNT and the IgM ELISA without detectable IgG antibodies. In the third period, which lasted from 8 until 28 d.p.i., pigs tested positive for IgM, IgG and IgG<sub>1</sub> antibodies. Starting from day 11, IgG<sub>2</sub> antibodies were detected. At day 35 IgM antibodies started to wane. In one of the animals infected with ITL/1/92, IgM was found until 91 d.p.i., but after

Table 3. Number and distribution of field sera of SVD outbreaks found positive by the IgM and IgG ELISA

Outbreak	Number of samples							
	Total	VNT negative			VNT positive			Distribution of positive sera on the farm
		IgM only	IgM and IgG	IgG only	IgM only	IgM and IgG	IgG only	
92-01	11	3	0	0	1	2	0	NT
92-02	5	NT	NT	NT	4	1	0	Localized
92-03	73	0	0	0	6	17	2	Localized
92-04	266	11	0	7	3	18	123	Randomly
92-06	363	3	0	0	5	70	10	Localized
94-02	22	0	0	0	3	13	1	NT

NT, Not tested.

infection with the other strains, no IgM was detected after day 42.

Using logistic regression (Genstat), we calculated the day at which 50% of the animals ( $D_{50}$ ) became positive or negative in a test. The  $D_{50}$  for appearance of neutralizing antibodies detected by the VNT was 5 days, and 4, 12, 12 and 24 days for the appearance of detectable antibodies in the IgM, total IgG, IgG<sub>1</sub> and IgG<sub>2</sub> ELISA, respectively. A  $D_{50}$  of 49 days was calculated for the disappearance of IgM antibodies (Table 2).

### Field sera

To check the specificity, 156 VNT-negative field sera were tested in the IgM, IgG, IgG<sub>1</sub> and IgG<sub>2</sub> ELISA, resulting in 4, 3, 15 and 11 positive samples, respectively, resulting in a specificity of 97, 98, 90 and 93%, respectively.

Table 3 shows the number of VNT, IgM and IgG positive sera found in the different outbreaks at the time of diagnosis. In farm 92-01, three sows and two piglets with clinical signs were seropositive. Two sows with old lesions on the nose were IgM and IgG positive, the third sow had fresh lesions and was only VNT and IgM positive. The sixth positive pig, a boar without clinical lesions, was positive only in the IgM ELISA. Farm 92-02 had the highest percentage (80%) of positive pigs with only IgM antibodies (4 out of 5 positive samples). VNT-negative sera from this farm were no longer available so only positive sera could be tested. On farm 92-03, 25 of 73 (34%) of the pigs tested positive; the positive sera mainly contained IgM and IgG antibodies. The highest number of

VNT-positive pigs with only IgG antibodies (123 of 266) was found on farm 92-04. On this farm 9 serum samples with low VNT titres (1.5–2 times the titre of RS 01.04.93), were negative in the IgM and IgG ELISA. These 9 sera were positive in the liquid phase blocking ELISA [11], and 8 out of 9 scored positive in the IgG<sub>1</sub> or IgG<sub>2</sub> ELISA, indicating an old infection. On farm 92-06 24% of the animals scored positive in one of the tests.

Sera from outbreak 92-01, 92-02, 92-03 and 94-03 were tested for IgA antibodies. In pigs with high IgG titres 16 out of 35 (46%) were IgA positive. Sera from outbreak 92-04 were tested in the IgG<sub>2</sub> ELISA, 153 out of 266 being positive. Fourteen of the 153 positive sera were negative in the IgG ELISA; 7 of these 14 were negative in the VNT, and 1 contained IgM.

On farm 92-02, 92-03, 92-04 and 92-06, the results of all tests were analysed as shown in Figure 2. On farm 92-02, 92-03 and 92-06, the positive pigs were clustered. On farm 92-02, 7 of 8 seropositive sows were located in one row, close to the seropositive boar. The eighth positive sow, positive in the VNT and IgM ELISA, was housed elsewhere in the same section. The results of the tests and the location of the seropositive pigs on farm 92-03 are shown in Figure 2. On this farm, two pigs that were only positive in the IgG ELISA were found in two separate pens. Thirteen of the other 14 positive pigs were housed in close proximity of these two animals. On farm 92-06, the infection was also localized in a limited number of pens. In two of the three sections in one building, all pigs were serological positive. In the third section of this building, no seropositive pigs were found. On farm 92-04, the seropositive pigs were more or less

	1 ◆	2 ◆
	2 -	1 ◆
	1 △	1 ◆
VNT negative		1△ 1◆
- IgM and IgG negative	2 ◆	2 ◆
	2 ▲	1 ◆
VNT positive	2 -	2 -
▲ Only IgM positive	2 -	2 -
◆ IgM and IgG positive	1 -	1 -
△ Only IgG positive	2 -	2 -
	2 -	1- 1▲
	2 -	1 -

**Fig. 2.** Distribution of the results of the VNT, IgM and IgG ELISA on serum samples collected in 21 pens of one stable of outbreak farm 92-03. In two other stables, all 19 samples taken from 13 pens were negative in all tests.

randomly distributed on the farm. Pigs with only IgM antibodies were found in 6 of the 10 sections, pigs with IgM and IgG antibodies were found in 4 of the 10 sections. In addition, pigs with only IgG antibodies were found in 6 of the 10 sections.

## DISCUSSION

Because it was suggested that the time of infection on the 1992 and 1994 Dutch SVD outbreaks might have been earlier than 28 days prior to diagnosis, we developed and evaluated five isotype specific ELISAs to detect antibodies against SVDV. The IgM and IgG ELISAs were used to estimate the time of virus introduction on those Dutch outbreak farms. For validation of the newly developed ELISAs, we used sera from pigs infected in the bulb of the heel, although this is probably not the natural infection route. Previous experiments showed that animals infected naturally by contact with a contaminated environment became infected the first day of contact, and neutralizing antibody titres were comparable to those found in animals infected in the bulb of the heel [4]. The day IgM was first detected was comparable to the results found previously [6]. In contrast to the previously described test [6], we were able to detect IgM antibodies beyond 25 d.p.i., which was the limit of detection in the previously described test. We used an antibody capture assay (ACA) for detection of both IgM and IgA, instead of an indirect test as described by Brocchi et al. [6]. The ACA

approach has among others been used for bovine rotavirus [14], bovine respiratory syncytial virus [15], pseudorabies virus [16] and influenza virus [17] and shown to overcome antibody competition between IgM or IgA, and IgG antibodies [14]. The design of the IgG, IgG<sub>1</sub> and IgG<sub>2</sub> ELISAs used in this study was comparable to the previously described IgG ELISA [6], and the IgG ELISA produced comparable results. Responses of total IgG, IgG<sub>1</sub> and IgG<sub>2</sub> all lasted until the end of the experiments. Table 2 shows the time at which the different tests scored positive after experimental infection. The fact that IgM antibodies could be detected for a longer period helps to estimate the initial infection date. On the other hand, the fact that one pig was still IgM positive 91 d.p.i. makes it difficult to determine the exact endpoint of the IgM response. Because of these individual differences in responses, we introduced the  $D_{50}$ , the day at which 50% of the animals became positive or negative. Using the  $D_{50}$  acknowledges the differences between the responses of individual pigs, and allows a more reliable estimate of the time of initial infection.

Being positive in the IgG ELISA and negative in the IgM indicated that the pigs had been infected more than 49 days ago (based in the  $D_{50}$ , Table 2). Farm 92-04 was suspected of having been infected for more than 3 months, therefore we looked for a method to estimate the time of infection beyond this period. The serum IgA response seemed a promising tool for the estimation of infections beyond 49 days, because in some pigs the SVD specific IgA antibody response seemed to last for approx. 150 days. The variation, however, in IgA responses between experimentally infected pigs was considerable. The results obtained on a few outbreak farms confirmed this variation. Although the IgA response could not be used to estimate the time that elapsed after infection, the long-lasting nature of the IgA response, however, was remarkable. IgA is specific for mucosal surfaces, so the differences between pigs might be caused by differences in the involvement of the intestinal mucosa. After oral [18] and intradermal infection into the bulb of the heel [2] SVDV can be found in faecal samples. After oral infection some pigs excreted virus in their faeces for 126 days [18], in contrast to the intradermally infected animals where virus could not be isolated after the 7th day [2]. Perhaps the duration of the intestinal infection is correlated with the height and length of the IgA response.

Based on the results of the isotype specific ELISAs on sera of the experimentally infected pigs and the

good specificity, it was decided to use mainly the IgM and IgG ELISA for the analysis of outbreak sera. Farms 92-01 and 92-02 had been detected because of clinical suspicion by the owner. These two farms had the highest proportion of IgM positive sera and had no sera in which only IgG was detected (Table 3), the introduction of SVD can be estimated between 12 and 49 days (based on the  $D_{50}$ ) prior to detection. On farm 92-01, blood samples were selectively taken from pigs with clinical symptoms, which almost certainly has caused a bias toward IgM antibodies. On farm 92-01 the IgG positive sera were still negative for IgG<sub>2</sub>, indicating that the infection had entered the premises less than 24 days earlier (based on the  $D_{50}$ ). On farms 92-03, 92-04, 92-06 and 94-02 sera containing only IgG were found indicating that the infection had entered at least 49 days before sample collection. Farm 92-04 had been infected for a longer time, because udder lesions were reported 2 months before the farm was detected by serological screening. Analysis of the sera in the isotype specific ELISAs unfortunately did not help to make a more precise estimate of the time of introduction. The finding that nine sera were negative in the IgG ELISA but positive in IgG<sub>1</sub>, IgG<sub>2</sub> and LPBE ELISAs might be explained by a decrease of titre in time. In the experimental infected pigs, however, high IgG titres were found even 160 days after infection. Previous experiments (unpublished data) have shown that the infection route (oral versus intradermal) influences the antibody titre found. So in pigs with low neutralization titres, the results found in the pigs experimentally infected intradermal in the bulbus of the heel are probably not representative. Low neutralization titres, however, were only found on farm 92-04, and therefore the results found in the experimentally infected pigs are valid for the pigs on the other outbreaks.

The maximum incubation period of 28 days mentioned in the EU directive, is used in that directive as the maximum period for which control measures should be applied, and might therefore better be termed 'safety margin'. Experimental infections have shown that the onset of clinical signs often occurs the day after infection but is sometimes completely absent [4]. The occurrence of the clinical signs, however, was not the problem in the Dutch outbreaks. The virus clearly produced clinical lesions, but the failure to detect the signs by the farmer and his veterinary practitioner caused the long detection periods. In the four outbreaks detected by serological screening, the detection period exceeded 49 days (based on the  $D_{50}$ ),

which also explains the high seroprevalence (> 24%) on these farms. When serological screening is used after the first detection of an outbreak then the 'safety margin' in the EU directive can probably be smaller, because 5 d.p.i. half of the infected pigs are already positive in the VNT. More data from other outbreaks are needed, however, to determine the maximum time needed to detect a 5% seroprevalence, which could then become the length of the 'safety margin'.

The results of this study show that  $D_{50}$  of IgM and IgG ELISA can be used very efficiently to elucidate the epidemiology of SVD. For efficient tracing, however, obligatory record keeping of contacts by the farmer, pig traders, lorry drivers and veterinarians, next to good identification and registration of farm animals should be introduced.

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