Pathogenesis, diagnosis and epizootiology of swine vesicular disease

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Pathogenesis, diagnosis and epizootiology of swine vesicular disease

Pathogenese, diagnose en epizoötiologie van vesiculaire varkensziekte

(met een samenvatting in het Nederlands)

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Index

Introduction 5

Chapter 1 21
Pathogenesis of swine vesicular disease after exposure of pigs to an infected environment. A. Dekker, P. Moonen, E.A. de Boer-Luijtze, C. Terpstra. Veterinary Microbiology 45 (1995) 243-250

Chapter 2 31
Detection of early infection of swine vesicular disease virus in porcine cells and skin sections. A comparison of immunohistochemistry, in-situ hybridisation and direct in-situ RT-PCR. W.A.M. Mulder, F. van Poelwijk, R.J.M. Moormann, B. Reus, G.L. Kok, J.M.A. Pol and A. Dekker. Journal of Virological Methods 68 (1997) 169-175

Chapter 3 41
Validation of a screening liquid phase blocking ELISA for serological examination of SVD. A. Dekker, P.L.J.M. Moonen, C. Terpstra. Journal of Virological Methods 51 (1995) 343-348

Chapter 4 49
Chimeric SVD viruses produced by fusion PCR: A new method for epitope mapping. A. Dekker, C.H. Leendertse, F. van Poelwijk, J.M.J. Rebel and R.J.M. Moormann. Journal of Virological Methods (accepted for publication)

Chapter 5 63
Validation of a monoclonal antibody-based ELISA to detect antibodies directed against swine vesicular disease virus. G. Chénard, M. Bloemraad, J.A. Kramps, C. Terpstra, A. Dekker. Journal of Virological Methods 75 (1998) 105 - 112

Chapter 6 75
Isotype specific ELISAs to detect antibodies against swine vesicular disease virus and their use in epidemiology. A. Dekker, F. van Hemert-Kluitenberg, C. Baars and C. Terpstra. Epidemiology and Infection (submitted)

Chapter 7 91
Swine vesicular disease singleton reactors: results of SVD antibody surveillance in the Netherlands 1993 - 1999. A. Dekker, P. Moonen, H. Swier, F. van Hemert-Kluitenberg, C. Terpstra

Summarising discussion 105

Dutch Summary 111

Curriculum vitae 115

Numbers and Facts 119

Introduction

Pathogenesis, diagnosis and epizootiology of swine vesicular disease

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History

Swine vesicular disease (SVD) was first observed in Italy in 1966, where it was clinically recognised as foot-and-mouth disease (FMD) (Nardelli et al., 1968). Physical and chemical analysis of the virus showed that it differed from FMD, vesicular stomatitis and vesicular exanthema viruses and that it was very similar to viruses belonging to the genus Enterovirus within the family of *Picornaviridae*. SVD virus (SVDV) was subsequently isolated in an FMD vaccine trial in Hong Kong in 1971 (Mowat et al., 1972). In 1972, SVD was diagnosed in Great Britain, Austria, Italy and Poland. Table 1 shows the countries that reported outbreaks of SVD. In 1975 the first case of SVD occurred in the Netherlands (Franssen, 1975). Seventeen years later the virus caused six outbreaks (Terpstra et al., 1995). The last three outbreaks in the Netherlands occurred in 1994.

Table 1: Year of last appearance of swine vesicular disease outbreaks in the world. Based on the FAO Animal Health Yearbook (1971 - 1996) and information obtained from the European reference laboratory for vesicular diseases in Pirbright (UK).

Europe	Year of last appearance
Austria	1979
Belgium	1993
Bulgaria	1971
France	1983
Germany	1985
Greece	1979
Italy	1999
Malta	1978
Netherlands	1994
Romania	1985
Russia	1975
Spain	1993
Switzerland	1975
Poland	1972
Portugal	1995
Ukraine	1977
Great Britain	1982
North and South America	
Bolivia	1991
Nicaragua	1986
Asia	
Hong Kong	1991
Japan	1975
Korea	1980
Laos	1991
Lebanon	1992
Macau	1989
Taiwan	1998

Recently only Taiwan and Italy have reported outbreaks of SVD, but the virus is probably present in other Asian and perhaps also in East-European countries. North and South America are considered free of SVD, although Nicaragua (1986) and Bolivia (1991) have reported outbreaks to the FAO (Table 1). Because SVD generally does not cause serious problems, the disease is probably underreported. Recent FMD outbreaks in Italy (1993) and Taiwan (1997) coincided with increased reports of SVD diagnoses in both countries. This suggests that farmers, who are familiar with the symptoms of SVD, do not report the disease until they think it might be FMD.

After 17 years of absence the Netherlands was confronted with six outbreaks of SVD in 1992. Although the actual number of outbreaks was low, and a protection zone of over 1300 km^2 was established, much larger than required by the European Union (EU) regulations (Figure 1), a ban on export of live Dutch pigs was implemented by the EU on 24 September 1992. Export of pig meat and meat products was only allowed from farms outside the protection zone (92/478/EEC). The export ban was lifted for farms outside the protection zone on 14 October 1992 and for the whole country on 15 November 1992 (92/495/EEC). Due to outbreaks in Italy and alleged exports of SVD infected pigs from the Netherlands, another export ban on Dutch and Italian pigs was implemented by the EU on 26 February 1993 which lasted until 27 March 1993 (93/128/EEC). Meanwhile a new directive, 92/119/EEC, for the control of certain animal diseases, including specific measures for SVD had been implemented,

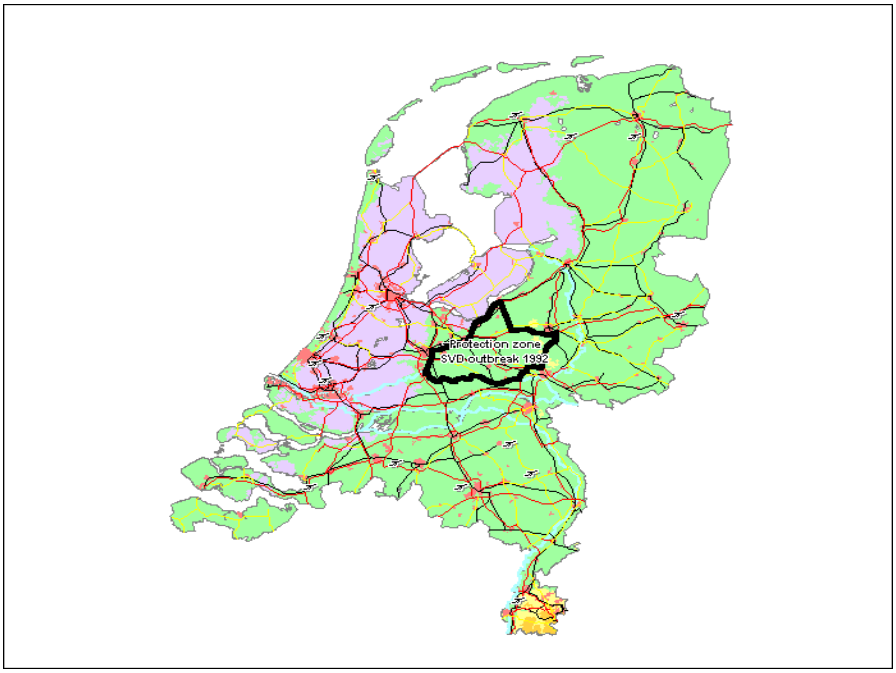

Figure 1: Map of the Netherlands showing the protection zone established in 1992

and several other commission decisions concerning SVD (93/177/EEC, 93/178/EEC, 93/179/EEC, 93/243/EEC) were taken in the first months of 1993. This large number of decisions illustrates the lack of knowledge on the disease transmission and prevalence of the disease, and the lack of trust in the serological tests used by the member states.

In 1992 and 1993 Italy was also experiencing SVD outbreaks and therefore monitored the pigs imported from other EU countries serologically and virologically. On several occasions SVDV was isolated from kidney and spleen suspensions collected at a slaughterhouse from finishing pigs originating from the Netherlands. The claim that the exporting country was responsible seemed valid, but no Italian pigs were tested. SVDV was often isolated from pigs negative for SVD specific antibodies and most pigs were kept for several days on the premises of the slaughterhouse. For this reason it was suggested that the pigs might have become infected at the premises of the slaughterhouse (Terpstra, personal communication). At that time it was not known how quickly a pig could become infected after contact with a contaminated environment. In previous experiments pigs had been infected by intravenous inoculation (Chu et al., 1979; Lai et al., 1979), intradermal injection in the bulbus of the heel (Burrows et al., 1974) or had been in contact with infected pigs. In these experiments SVDV could not be isolated from tissues of contact infected pigs before 2 days after contact.

The virus

SVDV is classified as an enterovirus within the family of *Picornaviridae*. The family comprises important animal and human viruses, e.g. FMD virus (FMDV), poliovirus, hepatitis A virus and a wide range of rhinoviruses. *Picornaviridae* are non-enveloped viruses and contain a single stranded RNA, with a positive polarity. The genome of SVDV consists of approximately 7400 nucleotides, which encodes a single polyprotein of 2815 amino acids (Inoue et al., 1989). This polyprotein is post-translationally cleaved into 11 proteins. Four of these proteins, 1A, 1B, 1C and 1D, form the virus capsid, one protein, 3B, is linked to the RNA and is therefore also a structural protein. The other proteins are involved in virus replication and host-cell shut off (Figure 2).

Several epitopes recognised by neutralising monoclonal antibodies have been mapped on the viral capsid (Kanno et al., 1995; Nijhar et al., 1999). In these studies the structure of polio-, human rhino- or Coxsackie B3 viral proteins was used as template to model the structure of SVDV, indicating a great structural homology between the different enteroviruses. Most but not all epitopes recognised by neutralising monoclonal antibodies had homologous sites on poliovirus (Nijhar et al., 1999).

SVDV is very resistant to environmental factors and many commonly used disinfectants. The virus remains infectious for months in carcasses and processed meat, e.g. salami or pepperoni sausages (Hedger and Mann, 1989). The virus can be grown on primary or secondary porcine kidney cells and a wide range of pig kidney-derived cell lines. SVDV can be differentiated from FMDV on

the basis of several physico-chemical properties (Table 2) and its inability to grow on primary bovine thyroid cells. SVDV can, however, be cultured in secondary lamb kidney cells (Dekker, unpublished observation). Infections in humans have been documented (Brown et al., 1976), and the virus is lethal to newborn mice (Nardelli et al., 1968). Within the group of enteroviruses only Coxsackie viruses can infect mice (Graves, 1973). Not only based on host tropism but also antigenetically SVDV is related to Coxsackie B5 virus (Graves, 1973; Brown et al., 1973). Based on this antigenic relationship, it was suggested that SVDV was a swine adapted Coxsackie B5 isolate (Graves, 1973). Sequence data show that SVDV has approximately 75 - 85 % nucleotide homology with Coxsackie B5 virus (Knowles et al., 1997), and phylogenetic analysis shows that SVD and Coxsackie B5 probably shared a common ancestor in the period between 1945 and 1965 (Zhang et al., 1999).

Table 2: Physico-chemical properties of SVDV and FMDV (Nardelli et al. 1968)

Property	SVDV	FMDV
Stability at pH 5	Stable	Labile
Stabilisation by 1M $MgCl_2$ at 50℃	Stabilised	Not stabilised
Ether-resistance	Resistant	Resistant
Sedimentation coefficient (S)	150	140
Buoyant density (g/ml)	1.34	1.43
Size (nm)	30 - 32	24
Morphology	Roughly spherical	Roughly spherical

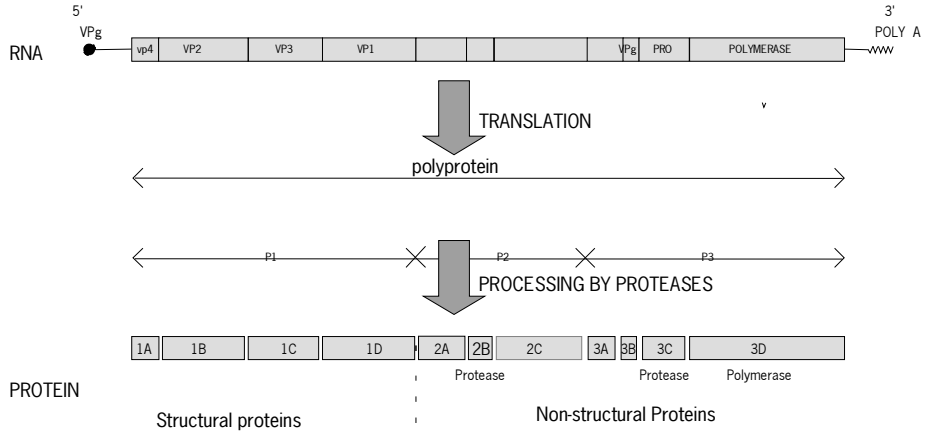

Figure 2: Genome organisation and expression of SVD proteins (Adapted from Rueckert, 1996).

SVDV is considered a single serotype, but isolates can be divided into four distinct phylogenetic groups by comparing monoclonal antibody reaction patterns and nucleotide sequencing of the 1D gene (Brocchi et al., 1997).

The disease

Clinical disease after an SVD infection is restricted to pigs. Not only Euro-Asian pigs but also American one toed pigs are susceptible (Wilder et al., 1974). Relatively high titres of SVDV have been detected in the pharynx of sheep kept in close contact with SVD infected pigs (Burrows et al. 1974b). In some of the contact sheep, neutralising antibodies were detected, indicating that the virus had replicated in the sheep. Strains of SVDV vary in virulence, and the disease may be subclinical, mild or severe. The latter is usually only seen when pigs are housed on a concrete floor in humid conditions (Kodama et al., 1980; Hedger and Mann, 1989; Kanno et al. 1999). In pigs infected with SVDV, vesicles appear around the coronary bands (Figures 3 and 4), on the skin of the metacarpus and metatarsus, and to a lesser extent on the snout, tongue and lips. Lesions are indistinguishable from those induced by an FMD infection. This is the main reason why SVD is considered important, and any outbreak of vesicular disease in pigs must be assumed to be FMD until proven otherwise.

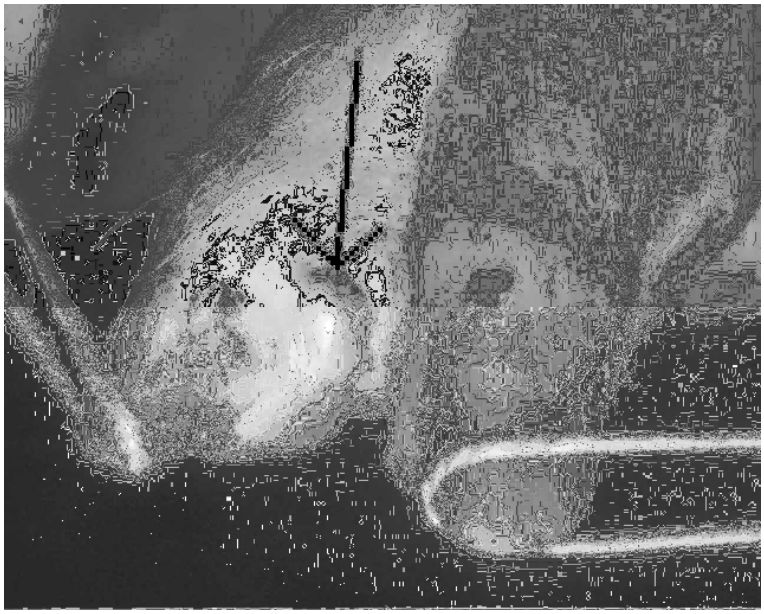

Figure : Vesicular lesion in the interdigital space. A vesicle on the coronary band extending to the bulb of the heel.

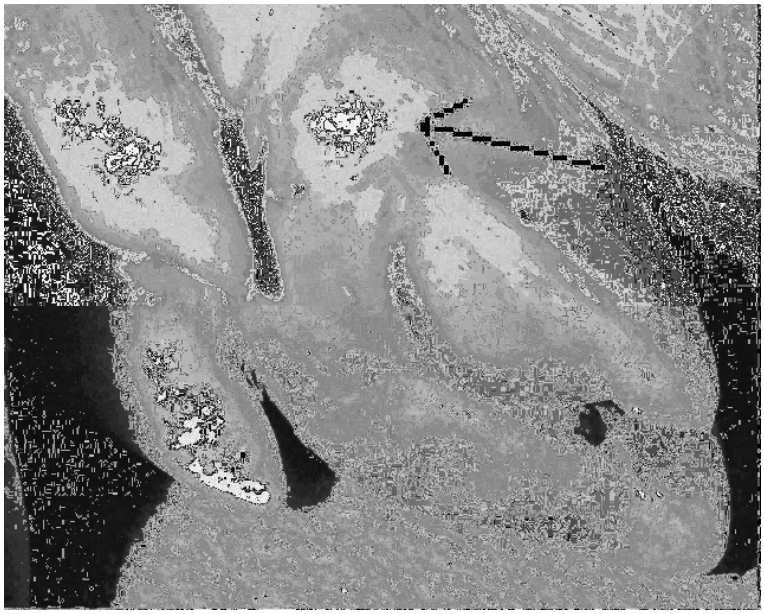

Figure 4: Vesicular lesion on the coronary band.

Sudden death, due to heart degeneration, which is often observed in young piglets affected with FMD, is not seen in SVD. In typical cases, lesions are first noticed at the junction of the heel and the coronary band. The whole of the coronary band may eventually be involved and the lesions may spread to the metatarsal and metacarpal regions. The horn and sole may be underrun so extensively that the claw(s) slough off. In lactating sows lesions on the udder and teats can also be seen. Occasionally, the skin of the thorax and abdomen is involved. Lesions in the mouth, on the lips and snout occur in up to 10% of the cases. Those on the snout are mostly on the dorsal face of the rostrum and may be haemorrhagic in appearance. Tongue lesions are transient and heal rapidly (Hedger and Mann, 1989). In experimentally infected animals a nonsuppurative meningoencephalitis may occur, but this does not result in signs of impaired central nervous system function (Chu et al., 1979).

Pathogenesis

It has been suggested that SVDV enters the pig through the skin or the mucosa of the digestive tract (Chu et al., 1979; Lai et al., 1979; Mann and Hutchings, 1980). Experimental SVDV infection can lead to clinical signs within 2 days and SVDV was isolated from a wide range of tissues (Burrows et al., 1974a; Chu et al., 1979; Lai et al., 1979). SVDV has a strong tropism for epithelial tissues, but also in the myocardium and the brain virus titres significantly exceeded those detected in plasma. So epithelial tissues,

myocardium and brain are probably the main sites of virus replication (Chu et al., 1979; Lai et al., 1979). Lymph nodes may also contain high titres of SVDV after experimental infection. It is not known, however, whether these tissues were merely positive, because of the drainage of virus or because of virus replication. Immunofluorescence staining and histological studies have demonstrated that SVDV first replicated in epithelial cells of the stratum spinosum of the epidermis, and then in a later stage also in cells of the stratum granulosum (Chu et al., 1979; Lai et al., 1979). Using the immunofluorescence test, no antigen was detected in the epidermis of the ventral part of the tongue. This is consistent with the fact that lesions in the thin epithelium on the ventral part of the tongue are not seen in SVD infections. The same applies to FMD infections. In-situ hybridisation has demonstrated that FMDV genome was present in a large number of epidermal tissues after FMD infection, irrespective whether or not it was a predilection site of vesicular lesions (Brown et al., 1995). This demonstrates that FMD grows in more cells than those previously identified, but the ventral part of the tongue was not studied. It can be expected that if an SVDV infection is studied using in-situ hybridisation also more tissues can be identified which can propagate the virus.

Diagnosis

After suspect signs of a vesicular disease in pigs, the farm should be treated as suspected of FMD until proven otherwise. Although virus isolation on IBRS-2 cells (De Castro, 1964) is considered the most sensitive method for virus identification (Dawe et al., 1973), the amount of virus in vesicular material is very high and can therefore easily be identified by antigen detection using an ELISA (Hamblin et al., 1984). In addition to IBRS-2 cells, SK6, PK-15 and primary or secondary porcine kidney cells are also susceptible to SVDV (Nardelli et al., 1968; Callens and De Clercq, 1999). High amounts (> $10^{3.5}$ plaque forming units) of SVDV induce nervous signs leading to paralysis and death in 1-day-old mice inoculated intracerebrally or intraperitoneally (Nardelli et al., 1968). Virus isolation takes one to several days, in contrast to antigen detection using the complement fixation test or ELISA, which can be performed within 4 h (Hamblin et al., 1984). Both the complement fixation test as well as the ELISA can be used on vesicular material from suspected pigs or they can be used to type virus isolated in cell cultures; the ELISA is much more sensitive than the complement fixation test. Using an RT-PCR (Lin et al., 1997; Núñez et al., 1998; Callens and De Clercq, 1999) viral genome can be detected. This technique sometimes produces false positive results due to contamination with the product of previous PCR reactions. RT-PCR, however, can be the test of choice if large numbers of faeces samples have to be tested. ELISA and virus isolation are currently still the preferred techniques for the laboratory diagnosis of suspected vesicular material (Anonymous, 1996).

In the aftermath of an outbreak detection of specific antibodies is essential to prove that no infected farms have been missed. After an SVDV infection, high titres of neutralising antibodies are found (Nardelli et al., 1968). Neutralisation

tests, however, are laborious, and therefore radial immunodiffusion and ELISA tests have been developed (Golding et al. 1976; Hamblin and Crowther, 1982; Armstrong and Barnett, 1989). Both tests are easier to perform than the neutralisation test, but produce more false positive results. Brocchi et al. (1995) showed that the specificity of the ELISA could be increased by using monoclonal antibodies (MAbs). The latter test therefore has been adopted as standard test by the Office International des Epizooties (Anonymous, 1996).

Epizootiology

An epizootiological field study in Great Britain revealed that the main source of infection was movement of pigs (48%), partly because infected pigs 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 (Hedger and Mann, 1989). The exceptionally high stability of the virus outside the host is the reason that indirect contacts, like transport vehicles or waste food, play an essential role in the epizootiology of SVD. Studies on SVDV transmission within a farm showed that spread from one pen to another may not occur in the absence of a shared open drainage system, or without frequent movement of pigs between pens; SVD is a 'pen disease' rather than a farm disease (Hedger and Mann, 1989). In the 1992 outbreaks in the Netherlands, the distribution of serologically positive pigs in both fattening farms and two of the three breeding farms confirmed the previous description, because seropositive pigs were mostly located in a few adjacent pens (Dekker and Terpstra, 1996). In one breeding farm, however, a more or less random distribution of seropositive pigs was found.

Because infected pigs are instantly culled it is not easy to study the transmission of SVD in the field. IgM and IgG ELISAs have been developed to study the time the virus was introduced (Brocchi et al., 1995). With these ELISAs, however, the exact time of introduction can not be assessed, when the infection has taken place more than 4 weeks before the collection of samples. Mathematical modelling might help, but to develop such a model information on the transmission within and between farms is needed. In contrast to FMDV infections in ruminants, persistence of SVD in infected pigs is not common, there is one report that the virus can be recovered up to 126 days after infection (Lin et al., 1998), but it has been difficult to reproduce these findings (Lin, peronal communication). FMDV can persist up to 3 years in cattle and up to 9 months in sheep. These FMDV carries are thought to play a role in the epizootiology of FMD (Salt, 1993). Recently there has also been a report on the persistence of FMDV in pigs (Mezencio et al., 1999).

Control and prevention

When SVD was first recognised, as a separately occurring disease, differentiating it from FMD, vesicular stomatitis and vesicular exanthema was not very easy. Therefore it was generally accepted that SVD could not be tolerated

in countries normally free from the other diseases. For this reason, SVD has been placed on the list A diseases of the Office International des Epizooties. SVD is therefore a notifiable disease in the European Union (EU) and in case of an outbreak, it is strictly controlled both by "stamping out" and restrictions on livestock movement. Stamping out involves slaughter and destruction of the infected herds, followed by tracing and surveillance of pigs on other premises that may have been exposed to infection. Following slaughter and disposal of pigs, the premises are cleaned and disinfected. Good disinfection is difficult especially in farms with a lot of crevasses in the floors and walls. Several cases of recurring infection have been reported after stamping out, and sometimes the infection only occurred in the pens where the infected pigs were housed the first time (Berlinzani, personal communication). The costs of control measures and trade restrictions can be very high. In 1993 the export of Dutch pigs was blocked for one month (decision 93/128/EEC), the cost of loss of export was estimated to be approximately 16 million Euro (Terpstra et al., 1995). Because of the economic losses, or the public health consequences, all list A diseases should be rapidly recognised, diagnosed and reported.

SVD, however, can be mild and the first cases may be missed. If the farmer or the veterinary practitioner does not recognise the disease, a large proportion of the pigs might become infected, and the disease might spread to other farms. Therefore serosurveillance is essential, especially in the aftermath of an outbreak, to detect subclinical- or undisclosed clinical infections, or to prove the absence of virus (Tokui et al., 1975; Hendrie et al., 1978; Pappous et al., 1980; Larenaudie et al., 1982). To prove freedom of SVD, the Netherlands has adopted a serosurveillance programme, in which farms are clinically examined and serologically tested three times a year. Currently, the costs of the SVD serosurveillance are over 3 million Euro per year. Italy is the only other country in the EU performing a serosurveillance programme, all the others rely on detection of SVD by farmers or veterinarians who recognise the clinical signs.

For the control of clinical disease SVD vaccines have been developed (Delagneau et al. 1974; Mowat et al., 1974; Gourreau et al., 1975; McKercher and Graves, 1976). Apart from monovalent SVD vaccines, also combinations with FMD (McKercher and Graves, 1976; Mitev et al., 1978) and recently a SVD sub-unit vaccine have been described. This latter vaccine was not very efficacious (Jiminez Clavero et al., 1998). Although the inactivated virus vaccines were efficacious in protection against clinical signs, it has not been evaluated whether it can reduce wild-type virus transmission. No SVD vaccine is commercially available and to date vaccination of pigs has not been undertaken in the field.

Scope of this thesis

The outbreaks of SVD in the Netherlands in 1992 and 1994 made us aware that although much information was available, there were considerable gaps in the knowledge on SVD pathogenesis and epizootiology. It was also recognised that serological tests capable of testing large numbers of sera were

hardly available. Therefore, these aspects of SVD have been studied and are part of this thesis. Epizootiological studies had shown that transport vehicles can be very important in the transmission of SVD, but it was not known how quickly virus could be isolated from pigs after contact with an SVD contaminated environment. Until then, in all experiments pigs had been infected by inoculation or by contact with inoculated pigs. In Chapter 1 experiments are described on the transmission of SVD by contact with a contaminated environment. From previous experiments (Chu et al., 1979; Lai et al., 1979) and the experiments described in Chapter 1, it was clear that SVDV could be found in several tissues shortly after infection. To be able to study the distribution of SVDV in tissues shortly after infection, we developed immunohistochemistry and in-situ hybridisation (Chapter 2).

At the start of the outbreaks in the Netherlands in 1992, only the liquid phase blocking ELISA (Armstrong and Barnett, 1989) and the virus neutralisation test (VNT) were available in our laboratory. The ELISA produced a high proportion (30%, Mackay personal communication) of false positive reactions when testing sera in the dilution described by Armstrong and Barnett (1989), for this reason we modified this ELISA (Chapter 3). In the aftermath of the 1992 outbreaks, approximately 70,000 sera have been tested, but in 1993, the Netherlands had to prove that pigs on farms exporting to other EU countries were free from antibodies against SVDV (decision 93/177/EEC). For this purpose over 700,000 samples were tested in 1993. Later, a nation-wide SVD serosurveillance programme replaced the export screening. In the serosurveillance programme approximately 700,000 sera per year had to be tested. Therefore, a sensitive and very specific test was needed. Brocchi et al. (1995) showed that these goals could be achieved by using monoclonal antibodies. For this reason we produced new monoclonal antibodies against SVDV (Chapter 4) and developed a monoclonal antibody based ELISA, which clearly is an improvement to the polyclonal antibody based tests (Chapter 5). For further characterisation of the newly produced monoclonal antibodies, we mapped the epitope regions recognised by seven of these monoclonal antibodies using chimeric viruses produced by fusion PCR (Chapter 4).

Because the cost for serosurveillance is high, it is essential to have information on the costs and benefits of the serosurveillance programme. For a good evaluation, information on the rate of virus transmission, expressed as R (number of new infections caused by one infection), should be available; not only, the R between pigs, but also between farms. An estimation of the latter might be possible using the data from the 1992 outbreaks. In Chapter 6 we describe the validation of isotype specific ELISAs for the detection of antibodies against SVDV. These ELISAs help to estimate the time of infection, and may help to estimate the reproduction ratio within and between farms.

The last chapter (Chapter 7) describes the singleton reactor phenomenon. Due to the large number of samples tested in the serosurveillance programme, considerable numbers of false positive reactions are found. These reactions are mostly limited to a single pig on a farm, and are referred to as singleton reactors.

Their reactions in the IgA and IgM ELISA and cross neutralisation with Coxsackie B5 are studied.

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Chapter 1

Pathogenesis of swine vesicular disease after exposure of pigs to an infected environment

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Abstract

The pathogenesis of swine vesicular disease (SVD) has been studied following a natural route of infection. In two experiments groups of ten and eight pigs respectively were introduced into a stable contaminated with SVD virus. At various intervals after stable exposure, pigs were killed and the amount of virus was determined in serum, vesicles (if present), spleen, kidney, and in seven lymph glands representing various parts of the body. One day after the pigs were introduced into the stable, five out of eight pigs were viraemic and virus could be isolated from various tissues. At 2 days after introduction, three out of four pigs killed had vesicular lesions on the feet. The tonsils of all pigs killed between 1 to 7 days after introduction into the stable were virologically positive. Four days after introduction 50% of the pigs were serologically positive and at 7 days all pigs had developed an antibody response. This study shows that contact with a SVD virus contaminated environment can be equally as infectious as injection or direct contact with SVD infected pigs, causing a rapid spread of the disease. Because the tonsil was shown to be highly efficient in trapping and growing circulating virus, we recommend that in addition to serological examination, virus isolation from pig tonsils should be used to study the epidemiology of SVD on farms where the infection is present.

1 Introduction

Four consignments of Dutch slaughter pigs were found to be positive for swine vesicular disease (SVD) by virus isolation from a mixture of spleen and kidney at an Italian abattoir early in 1993 (Terpstra et al., 1993). Since no other pigs were in the abattoir, the pigs were presumed to have been infected in the Netherlands. These events led the European Commission to impose a ban on the exports of Dutch pigs to member states. However, extensive clinical examination and serological testing on the farms of origin revealed no evidence of SVD infection. Furthermore, SVD virus could not be isolated from faecal samples collected at the collection centre where the pigs had been loaded for export. Because SVD virus was isolated only after the pigs had been held on the abattoir grounds for 2 days or more, suspicion arose as to whether the pigs might have been infected after unloading at the abattoir. However, experimental evidence to support this suspicion was scarce and until now there has been no evidence that pigs could become infected indirectly from the environment within 2 days.

SVD is a contagious disease. Pigs exposed to as little as $10^{1.5}$ pfu of SVD virus by intradermal inoculation of the feet or by scarification of the skin developed clinical disease (Burrows et al., 1974). Burrows et al. (1974) observed specific lesions at the site of inoculation 2 days after infection, and others have isolated SVD virus from pigs from 2 days up to 10 days after contact to infected penmates (Lai et al., 1979; Mann and Hutchings, 1980). In these experiments the pigs were infected by injection or were kept in direct contact with such pigs.

We studied the pathogenesis of a SVD infection in pigs using a natural route of infection, in order to explain the outbreaks at the Italian abattoir. We infected two groups of SPF pigs with SVD virus, removed them, did not clean the stable, and then introduced conventionally reared finishing pigs, to determine whether the infection could be transmitted indirectly within 2 days.

2 Materials and methods

2.1 Animal experiments

Groups of ten (experiment a) and eight (experiment b) conventionally reared finishing pigs weighing about 80 kg were introduced into a stable that had accommodated five to nine specific-pathogen-free (SPF) pigs. The SPF pigs were inoculated orally and also in bulbus of the heel (Burrows, 1966) with SVD virus isolate NL92-2 4 to 6 days before replacement by the finishing pigs. Four days before the SPF pigs were replaced by the finishing pigs the stable was no longer cleaned. The finishing pigs came from different pens on the same farm and were killed 1, 2, 3, 4, 7, 10, 15, and 23 days after being introduced into the contaminated stable. Samples of spleen, kidney, and tonsil were collected for virus isolation, as well as jejuno-ileo Peyers' patches, lymph nodulus (ln.) popliteus, ln. cervicalis superficialis ventralis, ln. mandibularis, ln. inguinalis superficialis, ln. mesentericus, and vesicles (if present).

2.2 Virus isolation

Samples of each tissue were prepared as a 1/10 (w/v) suspension in maintenance medium by mincing them with scalpel blades and grinding with sterile sand (using a pestle and mortar). After centrifugation the suspension was inoculated onto 25 cm^2 monolayers of IBRS-2 cells (De Castro, 1964). Virus was allowed to adsorb for 1 h, after which the monolayers were washed with maintenance medium, and 5 ml of fresh maintenance medium was added. During the 4 days of incubation the monolayers were inspected daily for evidence of cytopathic effect. In case of cytopathic effect or at the end of the observation period, the cultures were frozen, thawed, and tested in the IDAS-ELISA as described by Hamblin et al. (1984). Positive samples and serum were titrated on IBRS-2 cells in a plaque count assay. Tenfold dilutions of the sample were allowed to adsorb for 1 h on monolayers of IBRS-2 cells in a six-well plate. After 1 h, maintenance medium containing 1% methylcellulose was added. After 2 days of incubation the monolayers were rinsed with tap water, fixed, and stained with amido-black (0.1% amido-black in 1 M acetic acid, 0.09 M sodium acetate, 10% glycerol). Plaques were counted macroscopically. All incubations were made at 37°C in a humidified atmosphere containing 5% CO_2.

2.3 Virus neutralisation test

The virus neutralisation tests was performed as described before (Dekker et al., 1994). Briefly, 50 µl of serial twofold dilutions of each serum were mixed

with 50 µl of SVD virus suspension containing 30-300 $TCID_{50}$ and incubated for 1 h at 37°C in a humidified atmosphere with 5% CO_2. All dilutions were made in microtitre plates using Earle's minimal essential medium supplemented with 5% foetal bovine serum and antibiotics. After the addition of PK-15 cells (American Type culture collection, CCL33) to the plates and subsequent incubation for 4 days at 37° C, the monolayers were fixed and stained with amido-black and the test was read macroscopically for cytopathic effect. Titres were expressed as the reciprocal of the serum dilution that prevented virus growth for more than 50% in 50% of the replicate wells. In each test the virus suspension and a postinfection serum were titrated. Titres higher than 1.7 (^{10}log) were considered positive.

3 Results

In the first experiment, the nine SPF pigs used for contaminating the stable with SVD virus were killed 6 days after infection. At post-mortem examination, seven of these had vesicular lesions on their feet, and one had a vesicle on the nose. The five SPF pigs used for contaminating the stable in the second experiment were removed 4 days after infection and housed in another stable for collection of sequential sera. At 5, 6, and 8 days after infection one, three, and finally all five pigs respectively became serologically positive.

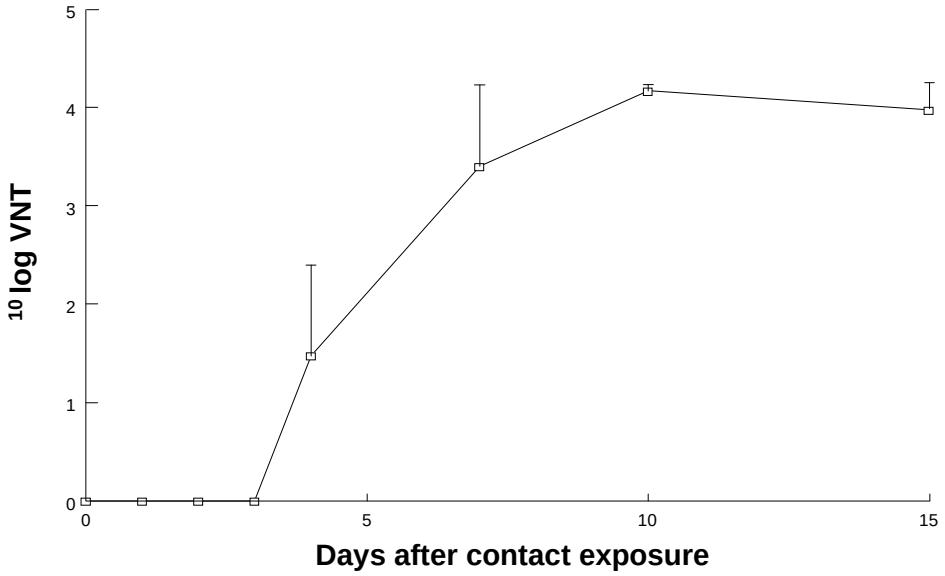

Figure 1: Development of neutralising antibodies in pigs exposed to an SVD-contaminated stable (mean titres including standard deviation)

Table 1: Virus titres measured in various tissues of pigs exposed to a stable contaminated with SVD.

Pig and exp. number	Day killed after exposure	Virus titre (^{10}log pfu/g)										
		Serum	Tonsil	Spleen	Kidney	Ln. mandibularis	Ln. cervicalis superficialis ventralis	Ln. inguinalis superficialis	Ln. popliteus	Peyers' patches	Ln. Mesentericus	Vesicle or lesion
1b	1	-[a]	5.08	2.64	2.18	2.61	4.00	2.80	+[b]	2.06	2.35	NV[c]
2b	1	3.05	4.18	-	-	4.11	-	+	+	2.99	-	NV
1a	2	5.40	5.96	4.45	3.65	4.09	4.08	3.94	NT[d]	3.24	3.39	8.48
2a	2	2.70	4.63	3.66	-	2.51	+	+	4.21	3.84	+	9.14
3b	2	-	5.03	1.70	-	-	-	-	+	2.35	+	NV
4b	2	4.91	6.05	4.30	3.18	3.45	7.24	3.28	7.09	3.38	3.46	9.82
3a	3	-	5.68	2.44	2.13	+	+	+	-	+	-	NV
4a	3	2.80	4.99	2.36	-	3.27	3.44	+	5.46	-	+	8.50
5b	3	3.83	5.80	2.74	2.24	4.42	3.33	5.54	5.07	3.50	2.51	NV
6b	3	3.72	6.96	3.47	2.64	4.28	6.15	5.28	7.21	3.51	2.35	9.64
5a	4	2.53	7.13	+	+	2.83	5.09	7.23	4.96	5.16	+	9.30
6a	4	-	5.53	-	-	3.82	2.75	6.33	NT	-	+	9.18
7b	4	2.50	7.20	+	+	3.73	6.03	5.64	1.70	3.59	+	9.15
8b	4	2.74	7.20	2.68	+	3.69	6.97	6.39	6.41	4.25	2.06	9.66
7a	7	-	6.00	3.20	+	3.68	6.02	5.47	1.70	-	1.40	9.56
8a	10	-	-	-	-	-	-	-	-	-	-	NT
9a	15	-	-	-	-	-	-	-	-	-	-	-
10a	23	-	-	-	-	-	-	-	-	-	-	NT

[a] - Negative by virus isolation
[b] + Positive by virus isolation but negative by plaque count (<1.40 10llog pfu/g)
[c] NV: no SVD lesion
[d] NT: not tested

At post-mortem examination, all pigs that had been in contact with the contaminated stable had scratches on the skin probably due to fighting. Five out of eight pigs killed 2 to 3 days after entering the contaminated stable had vesicular lesions on their feet; all pigs killed more than 3 days after entering had vesicular lesions on their feet and two had lesions on the nose (Table 1; animal 7a and 9a).

As early as 1 day after introduction high titres of virus were measured in various tissues (Table 1). However, no virus was isolated from pigs killed more than 7 days after entering the contaminated stable. The tonsils of all virologically positive pigs had virus titres that exceeded the virus titres in the serum (Table 1).

Pigs became viremic on day 1 to 4 after entering the contaminated stable. Already one day after entering the contaminated stable five out of eight pigs were viremic. Viremia ceased by day 7, probably due to neutralising antibodies (Figure 1). Neutralising antibodies were detected as early as day 4 in four out of eight pigs (titre $\geq$ 1.7) and by day 7 all remaining pigs were serologically positive (Figure 1).

4 Discussion

We studied the pathogenesis of a SVD infection in pigs using a natural route of infection, in order to explain the outbreaks at the Italian abattoir. We infected two groups of SPF pigs with SVD virus, removed them, did not clean the stable, and then introduced conventionally reared finishing pigs, to determine whether the infection could be transmitted indirectly within 2 days.

Burrows et al. (1974) observed primary lesions at the bulbus of the heel 2 days after inoculation and secondary lesions at 3 days. Intradermal inoculation in the abdominal region or the snout resulted in a generalised infection after 4 days. We detected SVD lesions only 2 days after the pigs had entered the contaminated stable. Considering that primary lesions after inoculation in the bulbus of the heel were found not before 2 days after inoculation (Burrows et al., 1974), the pigs in our experiments most likely were infected in the first few hours after entering the stable. The lesions therefore are likely due to infection of the epithelium at or near the coronary band, where the lesions occurred. Mann et al. (1975) showed that a SVD infection does not spread as readily when straw bedding is used instead of concrete floors. The pigs in our experiments were housed on a concrete floor, as at the Italian abattoir. Abrasion of the skin at the bulbus of the heel, the coronary band, or elsewhere, caused by fighting, is the most likely explanation for the rapid dissemination of the disease. Because virus was isolated from various other tissues, e.g. lymphnodes in the region of head and neck, the virus probably entered the host at multiple sites.

After contact with infected penmates, viremia starts 2-4 days later (Mann and Hutchings, 1980; Lai et al., 1979), and lasts up to day 6 (Lai et al., 1979). However, when SVD virus is injected in the bulbus of the heel, or intravenously, viremia is seen as early as 1 day after infection (Burrows et al., 1974; Lai et al., 1979). In our experiments five out of eight pigs were viremic 1 day after contact with the contaminated stable, and 14 out of 16 were viremic 2 days after contact.

On the fourth day after contact with the contaminated stable, all remaining animals had been viremic. In pig number 1a, 3a, and 3b in which the infection was still subclinical the day it was put to death and in which no viremia could be detected, spleen, kidney, and tonsil already contained considerable amounts of SVD virus (Table 1). This means that these animals must have been viremic, although it was not detected. Gradually viremia ceased when antibodies were detected 4 to 7 days after infection. In some animals, however, antibodies as well as virus were detected at the same time.

Burrows et al. (1977) detected the first antibody response in two out of eight animals 5 days after inoculating the coronary band. At 8 days all pigs were serologically positive. Intravenous inoculation resulted in an antibody response in three out of four pigs 3 days after inoculation, and all pigs were serological positive after 4 days (Burrows et al., 1977). The antibody response in the SPF pigs, infected orally and by injection, in our experiments is in complete agreement with the findings of Burrows et al. (1977). In our experiments six out of eight finishing pigs showed a serologic response 4 days after contact with the contaminated stable and all remaining pigs were serologically positive after 7 days.

Comparing pigs which were introduced into an environment contaminated with SVD, to pigs infected with SVD by injection or pigs in direct contact with SVD infected pigs, there is no significant difference in vesicular lesion formation, viremia, and antibody response. So it is evident that a contaminated environment is equally as infectious as injection or direct contact with infected pigs. This is probably because virus can enter at skin lesions caused by fighting or abrasions of the feet due to the concrete floor or both.

High titres of virus were measured in tonsils after pigs were introduced into a contaminated stable. This finding confirmed the findings of Chu et al. (1979) after intravenous inoculation, Burrows et al. (1977) after intradermal inoculation at the coronary band, and Burrows et al. (1974) after contact exposure to infected pigs. Because virus titres in the tonsil exceeded virus titres in the serum, we concluded that the virus must have grown in the tonsil. However, although the tonsil might have been an entry site of the virus, previous experiments have shown that large amounts ($\geq 10^{5.5}$ pfu) of SVD virus are needed to orally infect pigs (Burrows et al., 1974; Mann et al., 1975, Mann and Hutchings, 1980). Moreover, comparable large amounts of virus are needed to infect pigs via the surface of the tonsil (Mann and Hutchings, 1980). Therefore, it is more likely that the titres detected in the tonsil were the progeny of virus trapped from the blood circulation rather than from oral infection. Thus infected animals can be detected by virus isolation from the tonsil when they are still negative by serological test. This fact suggests that virus isolation from the tonsil can be used as an epidemiological tool to detect early infection on an infected farm. Together with serological tests it may help to elucidate the epidemiology of SVD on a farm where the infection is present.

In our experiments 80 of the 171 tissue samples tested had a virus titre equal to or higher than the titre found in the serum collected on the same day. Thus coronary band, tonsil, spleen, and kidney support SVD virus growth in vivo.

But the high amounts of virus found in lymph glands are probably due to high amounts of virus in afferent lymph. The ln. popliteus, ln. cervicalis superficialis ventralis, ln. mandibularis, ln. inguinalis superficialis, and ln. mesentericus receive lymph from tissues which support SVD virus growth in vitro (Mann and Hutchings, 1980; Habermehl et al., 1984). So from our observations no conclusion can be drawn about virus growth in lymphatic tissue in vivo.

Our experiments were designed to mimic to some extent the situation that may arise after long distance transports of pigs. Truck loads of about 200 slaughter pigs or up to 1000 piglets destined for breeding or fattening travel for more than 20 hours without being watered or fed. The pigs may originate from 20 different farms or more. During transport most animals already experience skin damages, but more injuries will occur after unloading, due to fighting for water and food. One infected animal with vesicular lesions (virus titres up to $10^{9.8}$ pfu/g) will quickly increase the amount of virus in the environment and disseminate the disease. When abattoir grounds are contaminated with SVD virus many pigs may be infected within hours after arrival. As shown in our experiments clinical signs may then develop within 2 days and the disease will spread rapidly. The outcome of our experiments therefore support the suggestion that the SVD virus isolated from pigs at the abattoirs in Nola and Avezzano were due to infection after arrival in Italy (Terpstra et al., 1993). Our results show that after a natural infection SVD virus can be isolated from different tissues, especially the tonsil, 1-7 days after infection; clinical signs of SVD may be observed after 2 days and neutralising antibodies can be detected as soon as 4 days after infection.

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Chapter 2

Detection of early infection of swine vesicular disease virus in porcine cells and skin sections. A comparison of immunohistochemistry, in-situ hybridisation and direct in-situ RT-PCR

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Abstract

Sensitive methods are required to study the early pathogenesis of swine vesicular disease (SVD). Therefore, two new methods, Immunohistochemistry (IHC) and in-situ hybridisation (ISH) were developed and tested for their specificity and sensitivity. With these methods the SVD virus (SVDV) infection in cytospins of primary porcine kidney cells and in frozen skin sections was investigated. Both IHC and the ISH showed a specific cytoplasmic staining, but the IHC detected more infected cells than the ISH. Furthermore, both IHC and ISH were capable to detect SVDV in skin sections 4.5 h after infection. It is concluded that IHC is the most suitable and simplest method to identify cells and tissues that support the initial replication of swine vesicular disease virus. However, IHC can only be applied to frozen sections, whereas ISH can also be used in paraformaldehyde-fixed tissues.

1 Introduction

Swine vesicular disease virus (SVDV) is classified as an enterovirus belonging to the family of the *Picornaviridae*. The single stranded RNA genome is 7401 nucleotides long and is of positive polarity (Inoue et al., 1989). SVDV is the causative agent of a highly infectious disease in pigs. Pigs of all ages are infected but they generally recover from the disease. However, the infection causes vesicular lesions on the snout, tongue, lips, the skin of coronary bands and the bulbs of the heels that are indistinguishable from those caused by foot-and-mouth disease. For this reason swine vesicular disease (SVD) has been added to the OIE-A list of diseases. An outbreak can have severe economic consequences. For example, the outbreak of SVD in 1992 in the Netherlands and a presumed outbreak in 1993 led to a complete ban on Dutch live pig exports which cost the Dutch Industry 36 million guilders (Terpstra et al., 1993).

The virus has strong tropism for epithelial tissues and it is suggested that SVDV enters the pig through the skin or the mucosa of the digestive tract (Chu et al., 1979; Lai et al., 1979; Mann and Hutchings, 1980). Dekker et al. (1995) have shown that pigs exposed to a SVDV contaminated stable can become viremic within 2 days. They used virus isolation to monitor the viral spread. After the start of the viremia most of the tissues examined became positive for SVD. Because of the rapid onset of the viremia it was not possible to identify the portal of entry. Others have used immunofluorescence staining to study the pathogenesis of SVDV (Chu et al., 1979; Lai et al., 1979). But in immunofluorescence studies it is difficult to see the morphological details of the tissues. So using virus isolation and immunofluorescence it is difficult to precisely identify the cells and tissues that are involved in the initial spread of SVDV.

In this study we developed three new methods, i.e. immunohistochemistry (IHC), in-situ hybridisation (ISH), and a direct in-situ reverse transcriptase polymerase chain reaction (direct in-situ RT-PCR) and tested their ability to detect SVDV shortly after infecting cells and tissues. We examined cytospins of

SVDV infected primary porcine kidney cells and frozen skin sections of a SVDV infected pig.

2 Material and Methods

2.1 Infection of cells

The UKG/27/72 strain of SVDV (Seechurn et al., 1990) was used to infect primary porcine kidney cells. Virus was allowed to adsorb for 1 h at 37 0C on a rotary shaker at a multiplicity of infection (m.o.i.) of 0.1, 1 and 2.5. After adsorption, virus was removed and the infected cells were cultured for 1, 2.5 and 4-5 h. Cells infected at 0.1 m.o.i. were also cultured for 15 h. Infected cells were harvested and resuspended in PBS. Cytospins were prepared (4 min 1000 rpm) on silanated slides (Perkin Elmer) using the Cytospin 3 (Shandon).

2.2 Animal experiment

Dutch Landrace pigs (10-weeks-old) were obtained from the specific pathogen free herd of the DLO institute for Animal Science and Health. Onto scarified skinparts of the back of one pig $10^{6.9}$ pfu of the NL92 strain of SVDV (Dekker and Terpstra, 1996) was administered. Virus was allowed to absorb for 1h before the suspension was removed. At 4, 5 and 8 h. after infection skin samples were collected for examination by IHC and ISH. The specimens were immediately frozen in liquid nitrogen and stored at -80℃.

2.3 Immunohistochemistry

Cytospins and frozen 6 μm-sections were air-dried and fixed in acetone for 10 min at room temperature (RT). Subsequently they were air-dried and stored at -20℃. Viral antigen in cells and skin sections was detected using a indirect immunoalkaline phosphatase (AP) assay. Sera were incubated for 30 min at RT and were diluted in a PBS solution containing 0.2% BSA. Briefly, sections were sequentially incubated with a purified polyclonal rabbit serum directed against SVDV (1:500, Hamblin et al., 1986), a biotinylated goat-anti-rabbit serum (1:500, Dakopatts E0432), and a AP-conjugated avidin-biotinylated enzyme complex (ABC, Vector Laboratories). After each incubation, sections and cytospins were rinsed for 5 min with running tap water. For colour development the AP substrate kit II (Vectorblack, Vector Laboratories) was used according to the manufacturer's protocol. Cytospins and tissues were counterstained with Mayer's Haematoxylin (Klinipath).

2.4 In-situ hybridisation

Two oligonucleotides VP1a and VP3, 30 and 29 bp in length, were selected in conserved regions of VP1 of VP3. Both oligonucleotides are complementary to the published SVDV sequences (Seechurn et al., 1990; Inoue et al., 1993) and are also complementary to the SVDV genome of the NL92

strain (F. van Poelwijk, personal communication). The antisense VP1 sequence 5'-ACT-CTG-CTC-ACA-ATG-GGA-CCG-GGA-CCC-CCA-3' is located at nucleotides 3161 - 3190 and the antisense VP3 sequence 5'-TAG-CAT-CGC-CTC-CTT-GCG-TGT-GGT-CGG-TG-3' is located at nucleotides 2218 - 2246. Both selected oligonucleotides were labelled at the 3' end using the tailing reaction with terminal deoxynucleotide transferase (Boehringer Mannheim), incorporating digoxigenin 11-dUTP and wer both used in the hybridisation reaction.

Cytospins and 6 μm sections were fixed for 5 min and 7 min respectively at RT in PBS containing 4% paraformaldehyde (freshly prepared). After fixation the cytospins were washed in PBS, dehydrated with ethanol, air-dried and stored at -80℃. Cytospins of cells and tissue sect ions were permeabilized with 1 and 10 μg proteinase K (Boehringer Mannheim) per ml Tris-HCl buffer (0.1 M, pH 8.0) containing 50 mM EDTA, respectively. Incubation with 10 μg/ml proteinase K for 15 min at 37℃ was optimal. After protease digestion slides were immersed for 2 min in DepC-water, dehydrated in ethanol (2 min sequential washes in 70% and 100%) at RT and air-dried.

Slides were prehybridised with 15 μl of hybridisation buffer [25% formamide, 4x SSC (1x SSC is 150 mM NaCl and 15 mM sodium citrate pH 7.0), 1 mM EDTA, fish sperm DNA (1 mg/ml), RNA (from yeast, 1 mg/ml), 5 x Denhardt's (50 x denhardt's is 1% polyvinylchloride, 1% pyrrolidone, 2% BSA) in 50 mM NaH_2PO_4\Na_2HPO_4 buffer pH 7.0.] and were covered with 15 mm round sterile coverslips. After 1 h incubation in a humid chamber at 37℃ the coverslips were removed by immersing the slides in 2x SSC. A 15 μl volume of hybridisation buffer with dig-labelled oligonucleotides (200 ng/ml) were applied to the slides. The coverslips were then sealed with nail-varnish and slides were hybridised for 16 h. in a humid chamber at 37℃. Po sthybridisation steps included removal of coverslips by immersing slides for 10 min in 2x SSC and two sequential washes in 2x SSC and 1x SSC for 10 min at 37℃.

Fab fragments of a anti-digoxigenin antibody conjugated with AP (1:200, Boehringer Mannheim) in a 100 mM Tris-HCl pH 7.5, 150 mM NACl, 0.2% BSA solution was used for 30 min at RT to detect digoxigenin hybridised probe. The AP substrate kit II (Vectorblack, Vector Laboratories) was used for colour development. After 20 min, the colour development was stopped by rinsing in tap water. The slides were counterstained with Mayer's Haematoxylin, dehydrated and coverslipped using DePeX mountain medium (Gurr, BDH lab., England).

3 Results

3.1 Immunohistochemistry

The specificity of the IHC method was assessed by omitting the primary antibody and performing the IHC on mock-infected cells and skin sections. No staining was observed in these negative controls (data not shown).
The sensitivity of the IHC method was analysed on cytospins of primary porcine kidney cells that were prepared at different timepoints after infection at different

m.o.i.s. The timepoint at which at which the first SVDV antigens could be demonstrated and the percentage of positive cells depended on the m.o.i. (Table 1). At a higher m.o.i. SVDV antigens were detected earlier and more cells were positive. The first viral antigen was detected at 3.5 h. after infection at 2.5 m.o.i. An example of cytoplasmic staining of viral antigens in cells, 16 h. after infection at 0.1 m.o.i., is shown in Figure 1A.

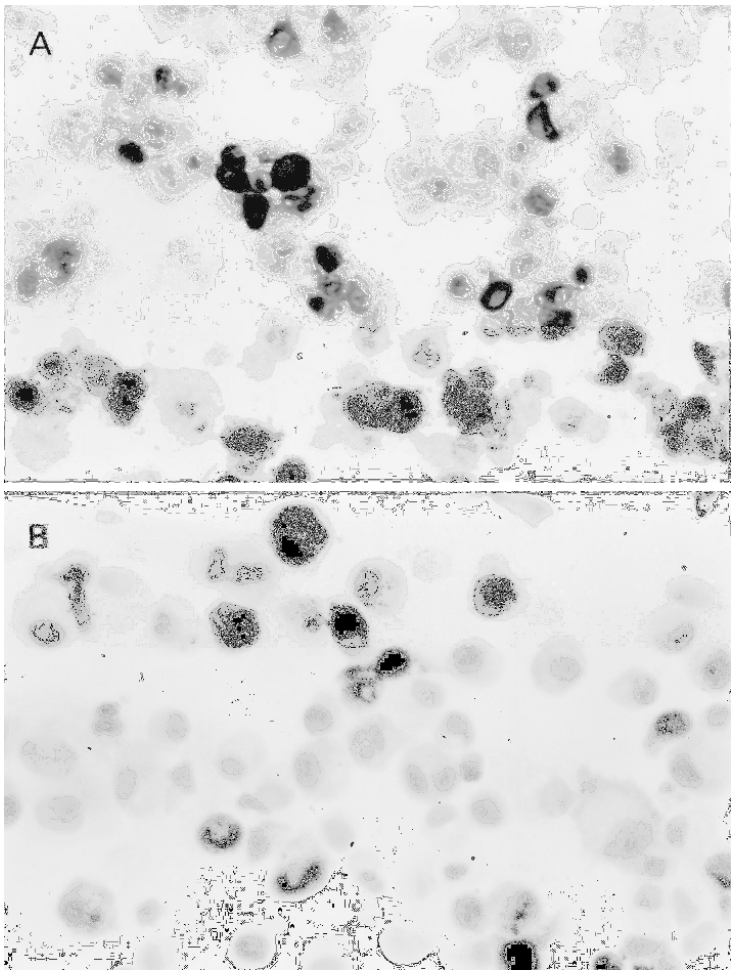

Figure 1: Primary porcine kidney cells 16 h after infection at 0.1 m.o.i. with SVDV strain UKG/27/72 showing cytoplasmic staining (black staining) with the IHC method (A) and with the ISH method (B). Photograph magnification x 320.

The IHC method was also suitable to detect early SVDV infections in tissue samples. In acetone-fixed frozen skin sections viral antigens could be demonstrated 4.5 h after infection. Immunostaining was seen in the epidermis and in the dermis (Figure 2A). Part of the positive cells in the dermis have the morphology of dendritic cells (arrows).

3.2 In-situ hybridisation

The specificity of the ISH method was examined by omitting the probes, by hybridising with a non-specific digoxigenin-labelled oligonucleotide probe and by performing ISH on mock-infected cells and skin sections. No ISH signals were observed in these negative controls (data not shown). Negative controls were included in every test.

The sensitivity of the ISH method was investigated on cytospins of infected primary porcine kidney cells. Positive cells were detected 16 h after infection at 0.1 m.o.i. and also a few positive cells were detected 5-6 h after infection at 1 m.o.i. (Table 1). An example of cytoplasmic ISH staining in cells, 16 h after infection at 0.1 m.o.i., is shown in Figure 1B.

Compared to the IHC method, the ISH method was less sensitive. The ISH method detected less positive cells and in contrast to the IHC method no positive signal was observed 5-6 h after infection at 0.1 m.o.i. (Table 1). However, the ISH method was sensitive enough to detect early SVDV infections in tissue samples. In paraformaldehyde-fixed frozen skin sections viral genomes could already be demonstrated at 4.5 h after infection. ISH staining was seen in the epidermis and in the dermis of skin sections (Figure 2B).

Table 1. Detection of SVDV in cells with IHC and ISH.

Hours after infection	Percentage of positive cells[a] with IHC after infection (m.o.i.)			Percentage of positive cells[a] with ISH after infection (m.o.i.)	
	0.1	1	2.5	0.1	1
2	0	0	ND	ND	0
3.5	ND	0	6.0	0	0
5 - 6	2.0	3.8	18.3	0	1.0
16	29.2	NA	NA	15.3	NA

ND, not done; NA, not applicable because of cell lysis.

[a] At least 300 cells were counted.

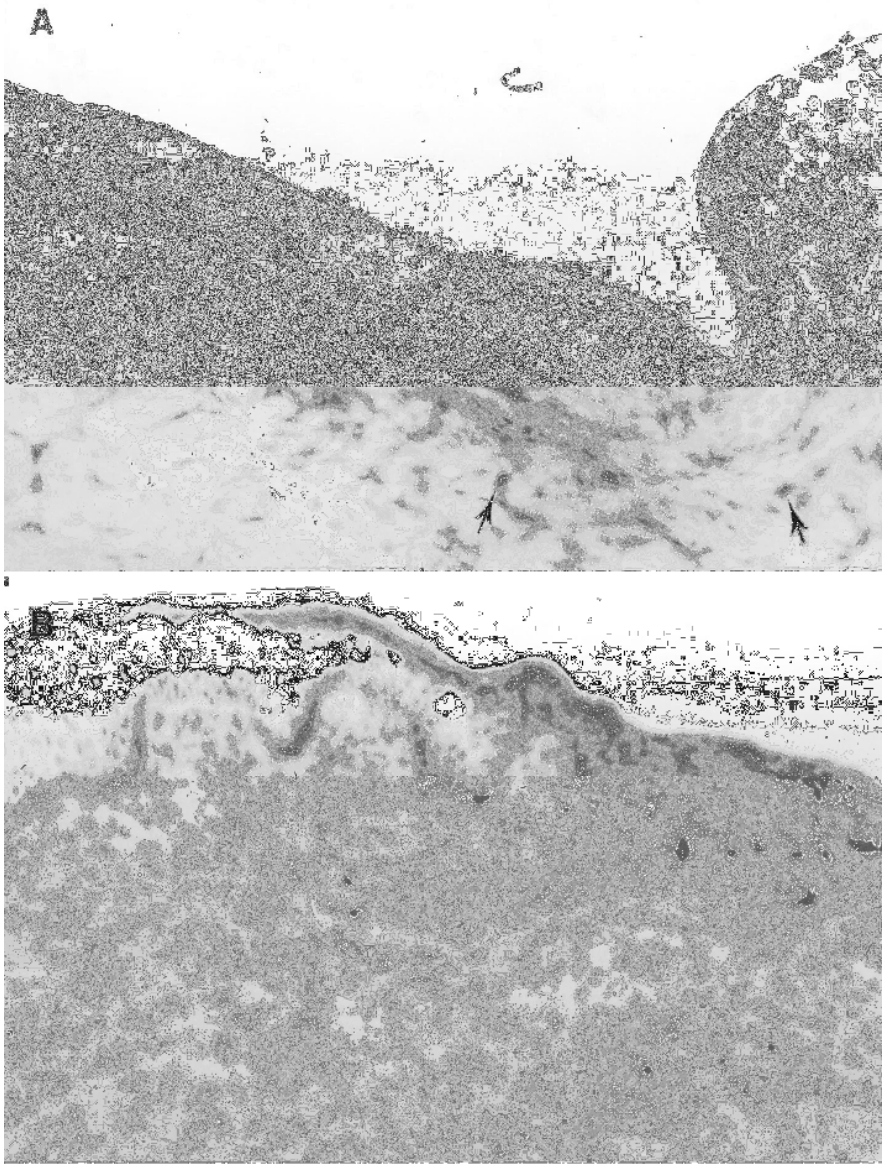

Figure 2: Detection of SVDV (black staining) in porcine skin sections, 4.5 h after infection with the NET/1/92 strain with immunohistochemistry (A) and with in-situ hybridisation (B). Positive staining could be seen in epithelial cells, cells of the basal membrane and in cells of the dermis, some of them with morphology of dendritic cells (arrows). Photograph magnification x 320.

4 Discussion

Two new methods, immunohistochemistry (IHC) and in-situ hybridisation (IHS) were developed and their ability to detect SVDV shortly after infecting cells and tissues was tested. It was found that both methods were suitable for SVDV detection in frozen skin sections and in cultured cells shortly after infection. Moreover, both techniques were suitable for detection of SVDV in other tissues such as tonsils and the skin of the coronary band. In addition, an attempt was made to develop a direct in-situ RT-PCR method, but it proved impossible to develop a functioning protocol.

A typical picornavirus multiplication ranges from 5 to 10 h and the synthesis of proteins starts 2-4 h (Rueckert, 1990). The length of the lag period lasts 2-4 h and can be reduced by increasing the m.o.i. (Baltimore et al., 1966). In agreement with this finding, it was observed that SVDV antigens could be detected earlier in cells infected at a higher m.o.i. Cells infected at an m.o.i. of 2.5 were positive after 3.5 h after infection, whereas cells infected at an m.o.i. of 1 were positive after 5-6 h. Thus, the IHC is probably able to detect SVDV antigen very quickly after the start of the protein synthesis. The earlier detection and higher level of detection indicates that our IHC method is more sensitive than our ISH method in detecting SVDV infection. In addition, using the simpler IHC method it is also possible to perform a double immunostaining, one for detecting SVDV antigens and a second for detection of a particular cellular marker. For instance, with double immunostaining the positive cells with dendritic morphology in the dermis (Figure 2A) and the positive epithelial cells in the stratum spinosum of the epidermis (Figure 2B) could be identified.

Unfortunately it was not possible to detect SVD antigens in paraformaldehyde- or formalin-fixed tissues using the IHC method. Antigen retrieval protocols using target unmasking fluid (Monosan) or microwave treatment using 0.01 M citrate buffer pH 6.0 could not restore the antigen recognition. Also a guinea pig serum and monoclonal antibodies directed against SVDV were not able to recognize SVDV antigens in paraformaldehyde- of formalin-fixed tissues after these treatments. Therefore, ISH may be used on paraformaldehyde- or formalin-fixed tissues and on archival paraffin-embedded tissues to detect SVDV.

Although, the IHC is more sensitive than the ISH, the results indicated that the ISH method is sensitive enough to detect SVDV infection. A widespread infection of SVDV in skin sections was detected 4.5h after infection using the ISH method. Moreover, ISH has been used successfully in pathogenesis studies of other Picornaviruses (Brown and Olander, 1995; Woodbury et al., 1995; Anderson et al., 1996).

It is assumed that the detection threshold for ISH is approximately 20 copies of the viral genome per cell. The (direct) in-situ RT-PCR method is believed to be more sensitive, because part of the viral genome is amplified. An attempt was mate to develop a direct in-situ RT-PCR method for SVDV, incorporating digoxigenin-11-dUTP during the PCR step, but it proved impossible to develop a functioning protocol. Although in-situ (RT)-PCR has

great potential for detection of viral infections with a low copy number (Nuovo et al., 1994; Gressens and Martin, 1994; Woodbury et al., 1995), many investigators have encountered problems with this new technique (Komminoth et al., 1994; Teo and Shaunak, 1995; O'Leary et al., 1996). The majority of these problems are due to degradation of target DNA or RNA, product diffusion and an improper balance between the fixation and subsequent permeabilisation of the tissues.

In conclusion, it was found that two sensitive methods are suitable for early detection of SVDV in tissues. The IHC method was optimised for acetone-fixed tissues and the ISH method for paraformaldehyde-fixed tissues. In future experiments double immunostaining against SVDV antigens and cellular markers can be performed. These experiments should reveal which cells and tissues are involved in the initial replication of SVDV.

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Chapter 3

Validation of a screening liquid phase blocking ELISA for serological examination of SVD

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Abstract

A direct liquid-phase blocking ELISA (LPBE) was developed for serological examination of swine vesicular disease (SVD). The sensitivity and specificity of the test were assessed on 272 and 365 sera collected on two farms where an outbreak had occurred. The specificity of the direct LPBE was higher than the specificity of the indirect LPBE. The European Community reference serum for SVD (RS 01.04.93), which has been adopted as the threshold for SVD serological examination in the European Community, had a mean titre of 2.19 in the neutralisation test. At a cut-off level of 2.0 in the neutralisation test, the sensitivity of the direct LPBE (screening at 1:432 final dilution) on the two farms was 90% and 99%, respectively. Based on these results, the screening dilution of the direct LPBE was adjusted to 1:160 final dilution, to obtain a sensitivity ≥98% on both farms. Regression analyses showed a good correlation between the virus neutralisation test and the direct LPBE (r=0.87). Compared to the indirect LPBE described before, the direct LPBE correlates better with the neutralisation test, has a higher specificity, and is more rapid. Because sera are tested in only one dilution, the test is highly suitable for the examination of large numbers of serum samples.

1 Introduction

After a 17 year absence of the disease, six outbreaks of swine vesicular disease (SVD) were diagnosed in the Netherlands in 1992. For serological examination of SVD various ELISA techniques have been developed (Hamblin and Crowther, 1982; Armstrong and Barnett, 1989). Initially, an indirect liquid-phase blocking ELISA (Armstrong and Barnett, 1989) was used. In this test an overnight incubation of serum and antigen at 4°C was changed to an incubation for 1 hour at 37°C on a rotary shaker (Luckham). Early in 1993, the European Community (EC) passed a regulation forcing the Netherlands to test herds for SVD antibodies before pigs could be exported to other member states. Since up to 12,000 samples a day had to be tested, a direct-type single dilution ELISA was developed to save time.

To evaluate the sensitivity and the specificity of the direct and the indirect ELISA, we compared the results of both tests with those of the virus neutralisation test on sera collected from two outbreaks, one on a breeding farm (92-04, farm A) and the other on a fattening farm (92-06, farm B).

2 Materials and methods

2.1 Sera

Sera (n=272) were collected from all sows and some piglets on the affected breeding farm (A) and from all pigs (n=365) on the affected fattening farm (B). A standard low positive serum (RS 01.04.93), distributed by the EC reference laboratory for SVD in April 1993, was used as a standard in all tests. This serum was adopted by the EC member states as the threshold for

serological tests for SVD used in the European community. Serum was taken from a pig immunized four times, 170 days after initial infection, and served as a positive control in the neutralisation test and both ELISAs. Negative control serum used in both ELISAs was obtained by pooling approximately two hundred 1ml samples of serum collected from SVDV serologically negative pigs.

2.2 Virus neutralisation test

The United Kingdom 1972 isolate of SVD virus, which was adapted to the PK-15 cell line (American Type culture collection, CCL33), was used in the virus neutralisation test (VNT) (Golding et al., 1976). Briefly, 50 µl of serial two-fold dilutions of each serum were mixed with 50 µl of SVD virus suspension containing 30-300 $TCID_{50}$ and incubated for 1 h at 37°C in an atmosphere with 5% CO_2. All dilutions were made in a microtitre plate using Earle's minimal essential medium supplemented with 5% fetal bovine serum and antibiotics. After PK-15 cells were added and further incubated for 4 days at 37° C in an atmosphere containing 5% CO_2, the test was read microscopically for cytopathic effect. Titres were expressed as the reciprocal of the serum dilution that prevented virus growth in 50% of replicate wells. In each test the virus suspension and the positive control serum were titrated. All sera were tested in a two-fold dilution series starting at 1.4 (1:25). Titres higher than 1.7 were considered positive.

2.3 Liquid Phase Blocking ELISAs

Elisa plates[1] were coated with a predetermined dilution of rabbit antibodies directed against SVD virus in phosphate buffered saline. The coating was incubated either stationary overnight at 4°C or 1 h at 37°C on a rotary shaker. Fifty microlitres of test serum diluted 1:216 in phosphate buffered saline containing 0.05% Tween 80 (PBST) were mixed with 50 µl of a predetermined dilution of SVD viral antigen (aiming for a maximum optical density of 1.5) and were incubated for 1 h at 37°C. Addition of the antigen made the final dilution 2.6 (1:432). After 1 h 50 µl of the serum-antigen mixture was transferred to a washed coated ELISA plate. After incubation for 1 h at 37°C, the plates were washed and incubated again for 1 h at 37°C with 50 µl of a predetermined dilution of pig-anti-SVD antibodies conjugated with horseradish peroxidase, diluted in PBST containing 5% fetal calf serum. After one more washing, 100 µl of chromogen substrate solution (0.04% orthophenylenediamine 0.015% H_2O_2) was added to each well. Colour was allowed to develop for 15 min. stationary at room temperature in the dark and then 100 µl 1M H_2SO_4 was added to each well to stop the colour development. Unless indicated, all incubations were made on a rotary shaker (Luckham). Each washing consisted of filling and emptying the plates six times with tap water containing 0.05% Tween 80.

[1] Costar Ltd.

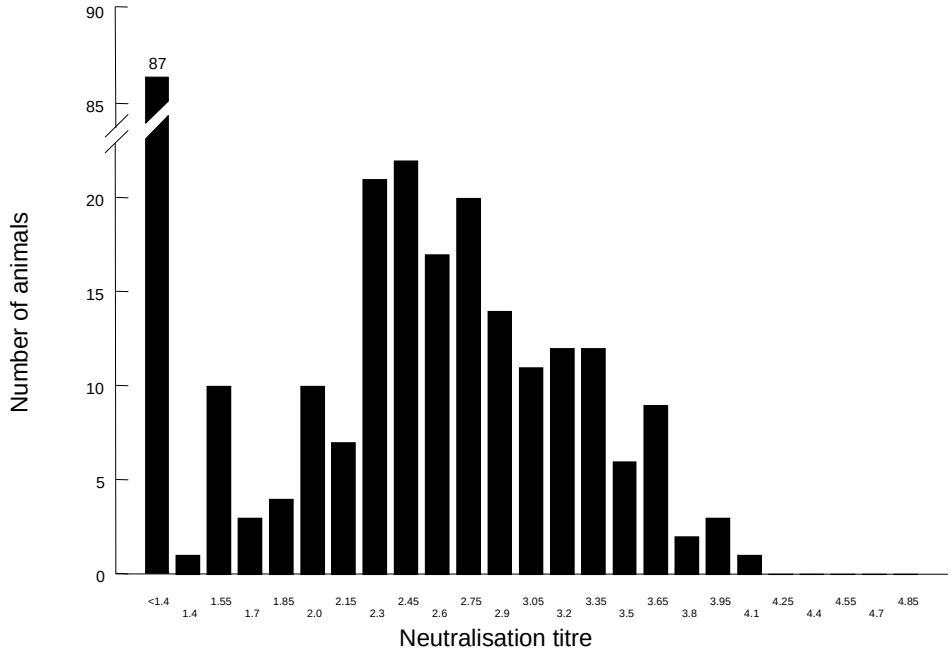

Figure 1: Distribution of neutralisation titres on breeding farm A

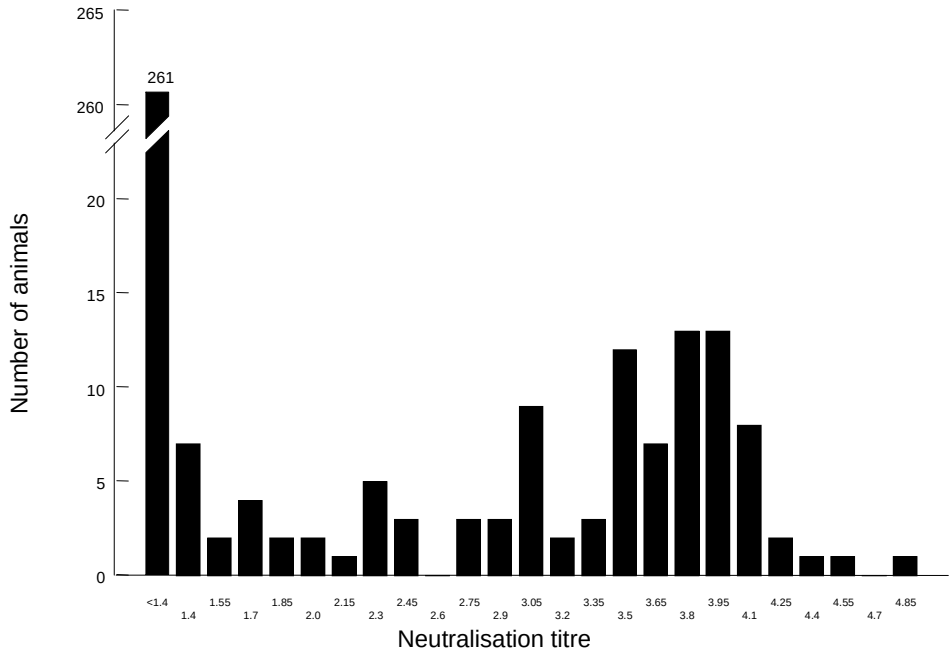

Figure 2: Distribution of neutralisation titres on fattening farm B

The plates were read at 492 nm, and sera were considered positive if the colour development (compared to the antigen control) was inhibited for more than 50%. An antigen control, a blank, a titration of the positive and a titration of the negative control serum were included on each plate.

The indirect LPBE was performed as described by Armstrong and Barnett (1989) with one modification. Instead of incubating sera and antigen overnight at 4°C as they did, we incubated these only for 1 h and at 37°C. Sera were tested at a final dilution of 2.5 (1:320). The screening dilution chosen for the indirect LPBE, was between the lowest titre found after experimental infection (2.95) and the highest titre found in 37 slaughterhouse sera collected in 1979 (titres up to 2.0).

3 Results

Two different screening dilutions were examined in the direct LPBE, testing 64 sera of farm A. The screening dilution (1:432 final dilution) which resulted in approximately the same sensitivity as was found by the indirect test was chosen.

Figures 1 and 2 show the distribution of the virus neutralisation titres of sera from farm A and B, respectively. The prevalence of positive pigs (neutralisation titre >1.7) on farm A was higher than on farm B (63% versus 25%). Within the group of seropositive animals, a mean neutralisation titre of 2.79 ± 0.52 was found for farm A, and 3.45 ± 0.66 for farm B, respectively.

Table 1 shows the sensitivity and specificity of the direct and the indirect LPBE as compared to two different cut-off levels in the virus neutralisation test. The 1.7 level is considered the highest negative value, the 2.0 level is just below the mean titre of the EC reference serum (2.19 ± 0.22, n=51). Table 2 shows the sensitivity of the direct LPBE as compared to the virus neutralisation test at different cut-off levels for farm A and B.

Table 1: Sensitivity (%) and specificity (%) of the direct and indirect LPBE at two different cut-off levels in the neutralisation test.

VNT cut-off level	Farm	Sensitivity LPBE (%)		Specificity LPBE (%)	
		direct test	indirect test	direct test	indirect test
>1.7	A	85	88	99	89
	B	97	93	95	92
>2.0	A	90	89	96	81
	B	99	98	95	92

Farm A: n=272 (171 sera VNT titre >1.7, 157 sera VNT titre >2.0)
Farm B: n=365 (91 sera VNT titre >1.7, 87 sera VNT titre >2.0)

Table 2: Comparison of the sensitivity (%) of the direct LPBE and the neutralisation test at different cut-off levels.

VNT cut-off level	Farm	Sensitivity LPBE (%)			
		LPBE cut-off level >1.9	LPBE cut-off level >2.2	LPBE cut-off level >2.5	LPBE cut-off level >2.8
>1.7	A	98	94	73	51
	B	99	97	95	93
>2.0	A	99	98	78	55
	B	99	99	99	98

Farm A: n=272 (171 sera VNT titre >1.7, 157 sera VNT titre >2.0)
Farm B: n=365 (91 sera VNT titre >1.7, 87 sera VNT titre >2.0)

A correlation coefficient of 0.87 between virus neutralisation test and direct LPBE was calculated for all 288 sera that had a titre higher or equal to the lowest detectable titre in the virus neutralisation test and the direct LPBE.

A mean titre of 2.18 ± 0.15 (n=12) was found testing the EC reference serum (RS 01.04.93) in the direct ELISA.

4 Discussion

To evaluate the sensitivity and the specificity of the direct and the indirect ELISA, we compared the results of both tests with those of the virus neutralisation test on sera collected on two farms where an outbreak had occurred.

There is a distinct difference between the distribution of neutralising antibody titres on both farms. On breeding farm A a mean neutralisation titre of 2.79 was found in the serologically positive pigs compared to a mean neutralisation titre of 3.45 on fattening farm B.

These differences have implications for the validation of both ELISA tests. The screening dilution of the direct test was chosen in order to obtain approximately the same sensitivity as the indirect test. By comparing the direct and the indirect test using sera of the two SVD-infected herds, we have been able to reach approximately the same sensitivity for both tests (Table 1). However, on farm A the specificity of the direct LPBE was higher than the specificity of the indirect LPBE (Table 1). The low specificity of the indirect test is in agreement with the results obtained in the United Kingdom, where over 30% of the sera tested were false positive in indirect LPBE (EC screening program; Mackay, personal communication, 1993).

Not only the specificity of the direct test was better compared to the indirect test, but also the correlation to the neutralisation test was improved. A correlation coefficient of 0.87 (Fig. 3) was found between the direct LPBE and

the neutralisation test, whereas Armstrong and Barnett (1989) found a correlation coefficient of only 0.66.

Because we wanted to achieve a higher sensitivity, the screening dilution was lowered to 1:160 (2.2) final dilution. Table 2 shows that a sensitivity of ≥94% was found at a cut-off level of 1.7 in the virus neutralisation test, and a sensitivity of ≥98% was found at a cut-off level of 2.0. Testing approximately 300,000 samples at this dilution, we found approximately 2% false positive samples (unpublished results). However, the EC ruled in 1993 that the EC reference serum must be positive in every test, we had to set our screening dilution at 1:100 (2.0) final dilution. The consequence of using a 1:100 dilution for screening was that approximately 5% false positive sera were obtained (unpublished results). All these sera had to be retested by the virus neutralisation test. A small increase of sample size, however, can compensate for a lower sensitivity in a test (Martin et al., 1992). Therefore cut-off levels of tests used for large-scale serological surveys, should be based on epidemiological and statistical data and not be based on the ability to detect a single low positive serum.

The direct LPBE is not only quicker and labour-saving, it also has a higher specificity and correlates better to the virus neutralisation test than the indirect test. It is therefore highly suitable for herd testing.

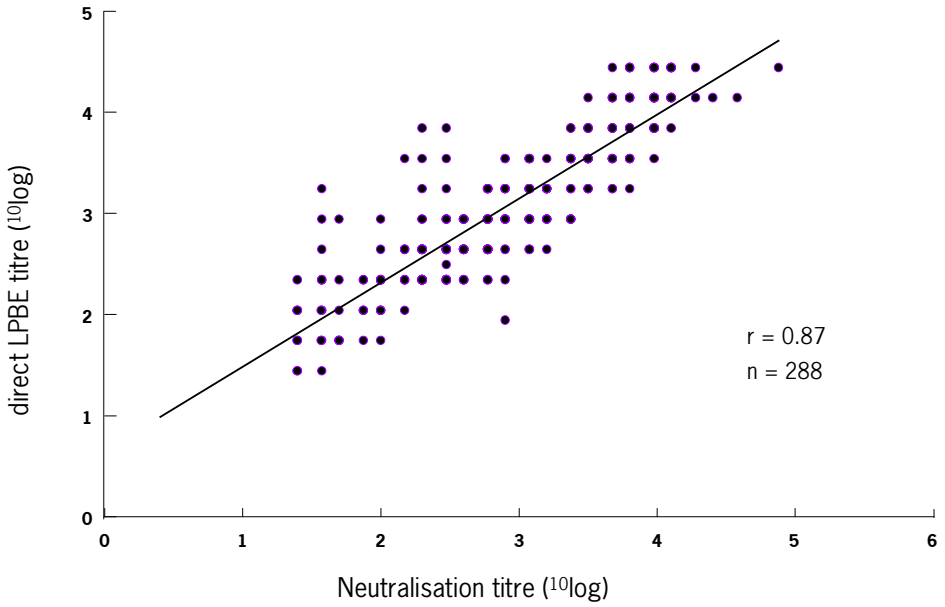

Figure 3: Correlation between virus neutralisation test and the direct LPBE

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Chapter 4

Chimeric SVD viruses produced by fusion PCR: A new method for epitope mapping

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Abstract

A new method of epitope mapping based on chimeric SVD viruses produced by fusion PCR. Seven out of Sixteen neutralising and non-neutralising newly produced monoclonal antibodies (MAbs) could discriminate between SVD isolate ITL/1/66 and NET/1/92. Using fusion PCR eight chimeric viruses were produced containing different supplementary pieces of the P1 region of both parent strains. Using these chimeric viruses we were able to map the epitope regions recognised by these seven neutralising and non-neutralising MAbs. This new method, using chimeric viruses produced by fusion PCR, is particularly valuable for the epitope mapping of non-neutralising MAbs.

1 Introduction

Swine vesicular disease virus (SVDV) causes a highly contagious disease in pigs characterised by vesicular lesions in the mouth and on the feet. These signs make the disease clinically indistinguishable from foot-and-mouth disease. For this reason swine vesicular disease (SVD) is classified as a list A disease by the Office International des Epizooties (OIE). SVDV is classified as an enterovirus within the family of Picornaviridae. It is a non-enveloped virus and consists of a ±30 nm capsid of icosahedral symmetry made up of 60 copies of each of the four structural proteins, VP1 to VP4 (Murphy et al., 1989; Rueckert, 1996). The RNA genome of SVDV is single stranded, of positive polarity, and contains approximately 7400 nucleotides (Inoue et al., 1989; Seechurn et al. 1990). SVD virus is antigenically closely related to Coxsackie B5 virus, a human enterovirus (Graves, 1973; Brown et al., 1973; Zhang et al., 1993; Zhang et al., 1999). It has been assumed that SVD may have arisen from an introduction of Coxsackie B5 into the pig population. But because sequence differences between Coxsackie B5 and SVDV are more extensive in non-structural regions compared to the structural part of the genome, another mechanism for the antigenic similarity cannot be excluded (Zhang et al., 1993; Zhang et al., 1999). Four major antigenic groups of SVD virus have been identified (Brocchi et al., 1997). The first group consists of the first SVD isolate ITL/1/66. The second group consists of viruses isolated in Europe from 1970 till 1980 with UKG/27/72 as the reference strain, which is also used in all serological tests in Europe. Group 3 consists of SVD strains isolated in Italy between 1988 and 1992, and group 4 consists of viruses isolated in different countries in Western Europe starting in 1992.

For SVDV serological tests, monoclonal antibodies (MAbs) proved to be essential to achieve high sensitivity and specificity (Brocchi et al; 1995, Chenard et al., 1998). Although a high specificity has been achieved, false positive sera are still encountered. In SVD serology these false positive sera are called "singleton reactor sera". Better understanding the antigenicity of the SVD virus particle may help to alleviate the problem of these so called "singleton reactor sera". Therefore new MAbs were produced and the epitopes recognised by these MAbs were mapped. Not only optimisation of diagnostic tests, but also

studies on viral pathogenesis, epidemiology and virus-host interactions may benefit from the knowledge of the epitopes recognised by the MAbs.

For epitope mapping of picornaviruses monoclonal antibody (MAb) resistant mutants are often used (Sherry and Rueckert, 1985; Crowther et al., 1993; Kanno et al., 1995). The disadvantage of this method is that only MAb resistant mutants can be produced against neutralising MAbs. Using monoclonal antibody resistant mutants, epitopes of non-neutralising MAbs can be linked to the epitopes found by neutralising MAbs (Brocchi et al., 1997), but if epitopes of non-neutralising MAbs can not be identified if they are located on a different position. Kanno et al. (1995), using monoclonal antibody resistant mutants, showed that three major epitopes could be identified on SVDV, which is similar to the situation in poliovirus. However, non-neutralising MAbs are also useful in serological tests (Chenard et al., 1998). Chimeric SVD viruses produced from a full-length infectious DNA copy of the viral genome have been used to identify virulence determinants (Kanno et al., 1999). We used a new approach to produce chimeric SVD viruses to map the epitopes of MAbs that could discriminate between two SVDV isolates. In contrast to Kanno et al. (1999) the chimeric viruses were not produced with a cloned infectious DNA copy of the viral genome, but by directly generating virus from full-length copy DNA (cDNA) produced by fusion PCR. Epitope regions recognised by MAbs, neutralising and non-neutralising, which discriminate between both parent strains, ITL/1/66 and NET/1/92, could be mapped using this new technology, showing that the method is valuable for the mapping of epitopes of non-neutralising MAbs.

2 Materials and methods

2.1 Viruses and cells

All SVD viruses, listed in Table 1, were obtained from the European reference laboratory for SVDV at Pirbright (United Kingdom), except strain ITL/1/66, which was obtained from Dr. E. Brocchi (Brescia, Italy), and NET/1/75, NET/1/92, NET/3/92, NET/1/94, and NET/3/94, which were isolated in our own laboratory. Coxsackie B5 isolates, A (942663) and B (Dekking) were kindly provided by Dr. T.J. Kimman (RIVM, Bilthoven, The Netherlands). All viruses were grown on IBRS-2 cells, using Eagle's medium supplemented with 5% foetal bovine serum (FBS) and antibiotics. When the cells displayed >90% cytopathic effect, usually after 1 day, the cell cultures were frozen and thawed. Before use in the ELISA, the virus suspension was clarified by centrifugation at 6000 g for 10 minutes. Before RNA isolation the clarified virus suspension was pelleted by centrifugation for 4h at 193,000 g in a Beckman Ti50 rotor. The pellet was also used for immunisation after a further purification by isopycnic centrifugation in cesium chloride (ρ=1.35 g/ml) for 16 h at 40,000 rpm at 4°C in a Sw50.1 rotor (Beckman). Transfection of full-length PCR products was performed on SK6 cells expressing T7 polymerase (SK6T7) (Gennip et al., 1999).

2.2 Production of monoclonal antibodies.

SVD isolate ITL/1/66 and UKG/27/72, belonging to the historic groups 1 and 2, and isolate NET/1/92 as the representative of one of the more recent antigenic groups (Brocchi et al., 1997) were used for immunisation. Mice were primed with approximately 100 µg purified SVD virus in complete Freunds adjuvant. Two weeks later the mice were boosted with purified virus in incomplete Freunds adjuvant. Mice with the highest neutralising antibody titres were selected for a final intravenous boost 10 days before the fusion. In fusion experiments numbers 124 and 143 the mice were immunised with strains UKG/27/72 and ITL/1/66 respectively. In fusion experiment number 145 strain ITL/1/66, UKG/27/72 and NET/1/92 were used alternately and finally boosted intravenously with a mixture of ITL/1/66, UKG/27/72 and NET/1/92. Fusion of spleen cells was performed according to the protocol of Fazekas de St. Groth and Scheidegger (1980).

Hybridoma culture medium was screened using the indirect ELISA described below. The supernatants were tested for reaction with ITL/1/66, UKG/27/72 and NET/1/92. Antibody-producing clones were subcloned at least two times by limiting dilution, the antibodies were concentrated by pressure dialysis (Millipore ultrafiltration system), and purified using a Protein A sepharose column (Pharmacia). An ELISA (Nordic) was used to determine the isotype of the MAbs.

MAb 5B7 was kindly provided by Dr. E. Brocchi (Brescia, Italy) and used as a positive control in the mapping experiments with the chimeric viruses (see below).

2.3 MAb screening ELISA

ELISA plates (Costar®) coated with rabbit antibodies directed against SVDV (Dekker et al., 1995) were used to trap a predetermined dilution of SVD virus. After washing the plate, supernatant of the hybridoma cell culture was added and incubated for 1 h at 37 °C. HRPO-labelled swine-anti-mouse (Dako P0260) was used to detect the MAb. All hybridoma cell cultures were tested using ITL/1/66, UKG/27/72 and NET/1/92 as antigen. Wells with an OD more than 1.0 were considered positive. Positive hybridoma cell cultures were tested the same way with the SVD and Coxsackie B5 strains shown in table 1. All strains were tested using an antigen dilution that produced an OD of approximately 1.5 when tested with polyclonal conjugated swine anti SVDV hyperimmune serum (Dekker et al., 1995).

2.4 Construction of chimeric mutant viruses by fusion PCR

Chimeric viruses were produced by fusing cDNA of the 5' end of the genome of one isolate to cDNA of the 3' end of the genome of the other isolate as outlined in Figure 1. First the 3' and the 5' end of the genome of both viruses were amplified in separate RT-PCR reactions. The primers were selected to

produce fragments that contained a small overlapping sequence. After purifying the PCR products from their primers the 5' end of the genome of one isolate was fused to the 3' end of the genome of the other isolate in a PCR reaction. The fused full-length DNA was amplified by PCR to produce enough cDNA to enable transfection into SK6-T7 cells.

In detail, RNA was isolated (Sambrook et al., 1989) and reverse transcription was carried out using M-MLV reverse transcriptase (Promega) and the oligo-dT primer (primer 10, Table 2) according to the instructions of the manufacturer. First-strand cDNA was amplified using Expand long template polymerase (Boehringer Mannheim) with the primers shown in Figure 2 and Table 2, using 25 cycles (standard: 10 sec 94°C, 30 sec. 55°C and 7.5 min. 68°C). The PCR fragments were purified using a "High pure" column (Boehringer Mannheim). Using the purified fragments in a standard PCR reaction (10 cycles) without primers resulted in a full-length chimeric infectious copy.

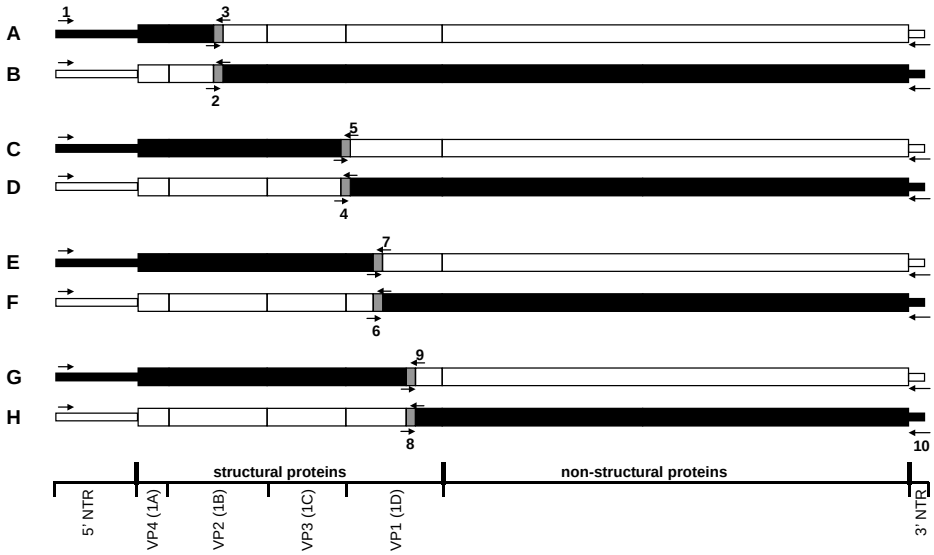

Figure 1: Schematic representation of the genome of the 8 chimeric viruses (A to H) produced by fusion PCR. Arrows indicate the location and orientation of the primers (1 to 10). The black region (■) represents the part originating from NET/1/92, the shaded region (▭) indicates the overlap region between NET/1/92 and ITL/1/66. The white region (▭) symbolises the part originating from strain ITL/1/66. The baseline represents the genome organisation of SVDV, showing the location to the viral capsid proteins VP1 to VP4 of relevance to the fusion sites in the chimeric viruses.
NTR = non-translated region.

Table 1 Reactivity pattern of the MAbs with different SVD and Coxsackie B5 virus strains as tested by ELISA

Group	Virus	143.1*	143.2*	143.3*	145.12*	145.8	145.11	145.2*	124.11	143.7	143.6	143.4	143.9*	143.10*	145.5	145.4	124.8
I	ITL/1/66	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+
IIa	BUL/2/71	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	UKG/27/72	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	FRA/1/73	-	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+
IIb	NET/1/75	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
	HKN/1/80	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
	HKN/11/81	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
	HKN/12/87	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
	HKN/24/88	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
III	ITL/A/89	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+
	ITL/1/92	-	-	-	-	-	-	+	+	-	-	-	+	+	+	+	+
IV	NET/1/92	-	-	-	-	-	+	-	-	+	+	+	-	-	+	+	+
	NET/3/92	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	SPA/1/93	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
	NET/1/94	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	NET/3/94	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	POR/3/95	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
	Coxsackie B5 (A)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	Coxsackie B5 (B)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Neutralisation	-	-	-	+	+	-	+	-	-	+	-	+	+	+	-	-
	ISOTYPE	IgM	IgM	IgM	IgG2b	IgG2b	IgG2b	IgG2b	IgG2a	IgG2b	IgM	IgG2b	IgG2a	IgG2a	IgM	IgG2a	IgG2a

*MAbs used for further characterisation by chimeric viruses

After this PCR reaction, the fusion products were amplified in a standard PCR reaction (25 cycles) using the SVDV specific 5` end forward primer (number 1, Table 2), containing a T7 promoter sequence, and a non-SVDV specific oligo-dT reverse primer (number 10, Table 2).

Transfection of the purified full-length PCR product into SK6T7 cells was done with Lipofectin (Gibco BRL) according to the protocol of the manufacturer. After 2 days, when the SK6T7 cells displayed cytopathic effects, the supernatant, containing the chimeric virus, was collected, and transferred to IBRS-2 cells to increase the virus titre.

Double-stranded DNA sequencing (ABI PRISM BigDye terminator cycle sequencing ready reaction kit, Perkin Elmer) was used on cloned RT-PCR products produced from the various viruses to determine the nucleotide sequences of the fusion regions of the chimeric viruses and the whole P1 region of both parent strains. Data collection was carried out with ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).

Table 2: Sequences of the 5' and 3' end primers used for the fusion PCR.

Primer	Sequence (5' → 3')	Length	Position nucleotide
1	CCCCTGCAGATC<u>TAATACGACTCACTATA</u>GTTAAAACAGCTTGTGGGTTGTT*	52	-30 till 22
10	AGATCTGCAGAAGCTTCGATCG$(T)_{40}$	62	Poly-A tail

*T7 promoter sequence is underlined

2.5 Virus neutralisation tests

The virus neutralisation test was performed as described by Dekker et al. (1995), with the exception that IBRS-2 cells were used instead of PK-15 cells. The neutralising capacity of the MAbs was tested against the three isolates, ITL/1/66, UKG/27/72 and NET/1/92, used for immunisation. The MAb was titrated starting with a concentration of 100 µg/ml of purified MAb.

3 Results

3.1 Specificity of the MAbs.

Sixteen MAbs were obtained which showed an OD >1 when tested in the ELISA with one of the three antigens (ITL/1/66, UKG/27/72 or NET/1/92) used for immunisation. The reaction of the MAbs with different SVD strains, isotype and neutralisation test results are shown in table 1. Several MAbs could discriminate between two viruses, a total of seven MAbs could discriminate between SVDV isolate ITL/1/66 and NET/1/92. Therefore were these two isolates selected to produce chimeric viruses.

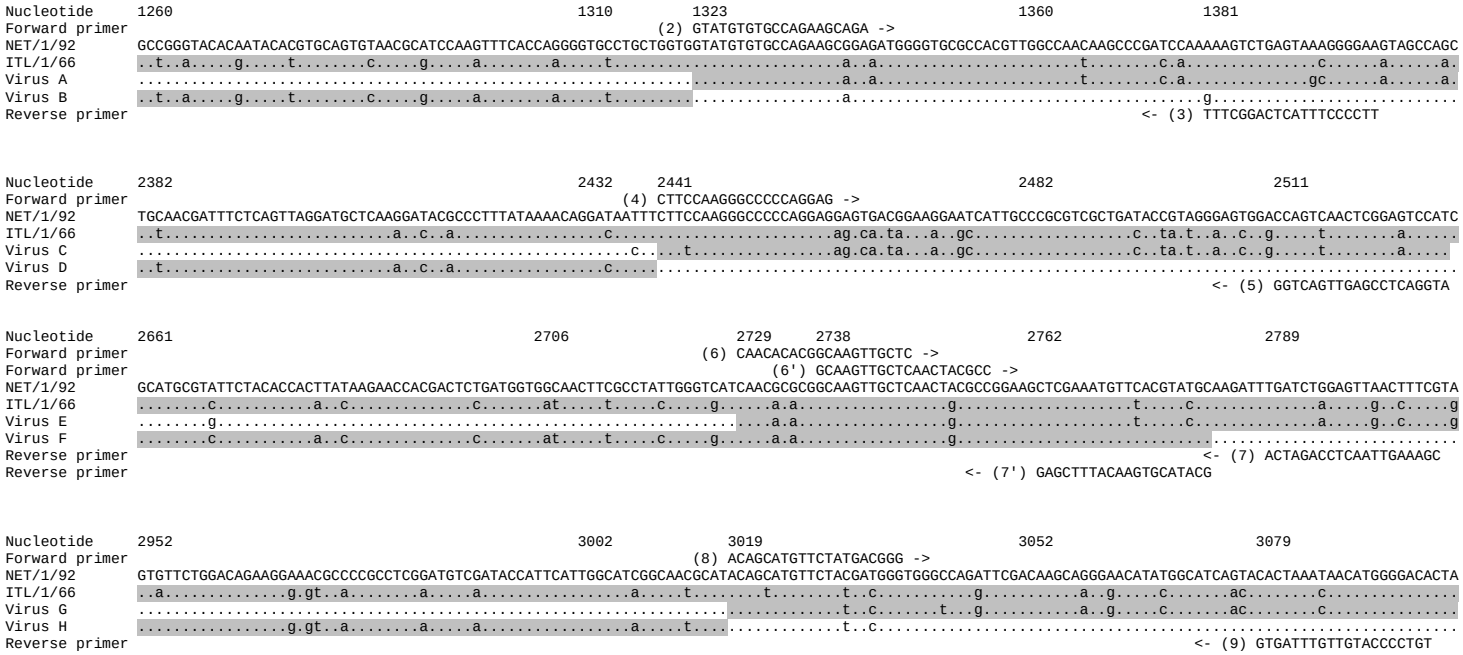

Figure 2: Sequence comparison of parent SVDV strains and the chimeric viruses. The numbers, locations and sequences of the forward and reverse primers are indicated. The parts of the genome originating from strain ITL/1/66 are shaded in grey. The unshaded nucleotides originate from strain NET/1/92. The arrows indicate the direction of amplification. The numbers on the top line represent the nucleotide number in the SVDV sequence.

3.2 Production of full-length chimeric virus.

Using fusion PCR we were able to produce full-length infectious chimeric viruses by exchanging genomic regions of SVDV ITL/1/66 and NET/1/92. The primers for the first PCR were chosen in such a way that an overlapping area of approximately 75 to 90 nucleotides was present in the fusion PCR reaction (Figure 2). All but one chimeric virus were produced with the initial set of primers. Only for virus F we used a new set of primers, 6' and 7', because we were not able to produce infectious virus using primers 6 and 7. In this new set of primers only an overlap of 45 nucleotides was present. The chimeric viruses produced were stable, gave cytopathic effects in IBRS-2 cells as quickly as the parent strains with a comparable plaque size (data not shown). Figure 2 shows 150 nucleotides of each chimeric virus of the area bordering the fusion. Of the total number of 1200 nucleotides shown in Figure 2, only 11 did not match with one of the parent strains. Four of these mismatches were introduced by the primers used for amplification: nt 1340 in virus B, nt 3023 in virus G and nt 3032 and 3035 in virus H. The mismatches introduced by the primers did not give rise to amino acid substitutions. Only 3 mismatches in these 1200 nucleotides shown gave rise to amino acid changes, in virus A at position 1393, in virus B at position 1381 and in virus G at position 3043. The nucleotide substitutions gave the following amino acid changes; In virus A an asparagine to serine, virus B a lysine to arginine and in virus G an alanine to valine. The other 4 remaining mismatches, nt 2438 and 2444 in virus C, nt 2669 in virus E and nt 2954 in virus H, did not give rise to amino acid substitutions.

Table 3**:** Reactivity pattern of the discriminating MAbs with the chimeric viruses

MAb	ITL/1/66	NET/1/92	Chimeric virus							
			A	B	C	D	E	F	G	H
5B7	-	+	-	+	+	-	+	-	+	-
143.9	+	-	+	-	+	-	-	+	-	+
143.10	+	-	+	-	+	-	-	+	-	+
143.1	+	-	+	-	+	-	+	-	+	-
143.2	+	-	+	-	+	-	+	-	+	-
143.3	+	-	+	-	+	-	+	-	+	-
145.2	+	-	+	-	+	-	+	-	+	-
145.12	+	-	+	-	+	-	+	-	+	-

3.3 Epitope mapping with chimeric viruses.

The chimeric viruses were used to determine epitopes recognised by the MAbs reacting with ITL/1/66 and not with NET/1/92. MAb 5B7 was used as a positive control; the epitope recognised by this MAb has been mapped on amino acid 163 of VP2 (Nijhar et al., 1999). Based on the reactions shown in table 3 and the known fusion region, MAbs 143.9 and 143.10 both react with the N-terminal part of VP1 of isolate ITL/1/66, between amino acids 5 and 87. In this region 7 amino acids differed between strain ITL/1/66 and NET/1/92 (Figure 3). MAbs 143.1, 143.2, 143.3, 145.2, and 145.12 all reacted with virus ITL/1/66, A, C, E and G. The epitopes recognised by these MAbs, therefore, must be located at the C-terminal part of VP1 of isolate ITL/1/66 between amino acids 199 and 283. In this region 5 amino acids differed between strain ITL/1/66 and NET/1/92 (Figure 3). None of the 7 discriminative MAbs were found to react either with VP2, VP3, VP4, or the central part of VP1 (between amino acids 88 and 198). Based on our experiments the epitope of MAb 5B7 was located between amino acid 143 of VP2 and amino acid 216 of VP3, which corroborates with the location (amino acid 163) found by Nijhar et al. (1999).

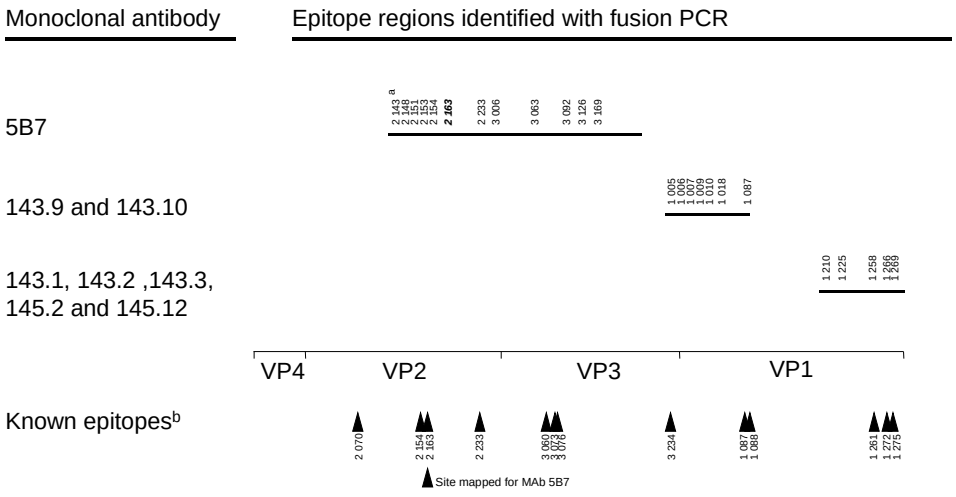

Figure 3 Epitope regions identified with fusion PCR.
[a] The amino acids which are different between strain ITL/1/66 and NET/1/92.
[b] Amino acids previously identified to be important in epitopes recognised by neutralising antibodies (Nijhar et al., 1999).

4 Discussion

4.1 Classification of SVDV isolates.

The grouping shown in Figure 1 corroborates with the findings of Brocchi et al. (1997) with one exception; we could clearly distinguish between European isolates from the early seventies and isolates from Hong Kong. This difference was also observed by Zhang et al. (1999) using nucleotide sequencing. SVDV strain NET/1/75, however, is clustered with the Hong Kong isolates. This outbreak might be due to introduction from Asia, because it was caused by feeding kitchen waste food from a NATO base nearby (Franssen, 1975). By sequence comparison, however, strain NET/1/75 is clearly linked to the European outbreak strains from the same period (Zhang et al., 1999).

In 1992 several outbreaks occurred in the Netherlands within a 500 m radius. One of the isolates, NET/1/92, reacted differently with some of the MAbs, compared to isolates NET/2/92 (data not shown) and NET/3/92, an observation already made previously by Brocchi et al. (1997). Outbreaks NET/1/92 and NET/2/92 were less than 30 meter apart. Both examples NET/1/75 and NET/1/92 show that using MAbs to study epidemiological links can be very helpful, but results are sometimes at variance with nucleotide sequencing and epidemiological findings. So for the identification or the exclusion of an epidemiological link on the basis of the MAb pattern, a previous characterisation of the MAbs, aimed at the identification of the epitopes and most important the stability of the epitopes, is essential.

4.2 Epitope mapping.

Epitopes recognised by neutralising MAbs can be mapped by the production of MAb resistant mutants, but it is not possible to produce MAb resistant mutants against non-neutralising MAbs. A long distance PCR spanning the entire genome of a picornavirus, resulting in infectious virus, has been described previously (Lindberg et al., 1997). Based on this example we developed a new method for epitope mapping, of particularly non-neutralising MAbs, using chimeric viruses produced by fusion PCR. Seven out of sixteen of the newly produced MAbs could discriminate ITL/1/66 from NET/1/92 (Table 1), therefore, chimeric viruses were made using these two strains. We constructed eight replication competent chimeric viruses containing different parts of the P1 of each strain.

In theory, a mixture of two chimeric viruses should be produced after fusion PCR, one containing the ITL/1/66 sequence and one containing the NET/1/92 sequence in the fusion region. From each of the eight chimeric viruses up to 23 different plaques per virus strain were picked and sequenced. In all cases we found only one sequence in the fusion region and in seven out of eight strains it was the sequence of the virus used to produce the 3' end. In these seven chimeric viruses it was the virus which had the shortest extension in the fusion reaction. Incomplete production of double stranded DNA by the

polymerase might have been the reason. The longer the part that has to be extended, the higher the chance of incomplete double stranded DNA production.

Using the chimeric viruses we were able to map the epitope regions of 7 of the 16 newly produced MAbs. Amino acid 163 of VP2 has previously been found essential in the epitope recognised by MAb 5B7 (Nijhar et al., 1999). This corroborates with the epitope region found in this study, demonstrating the validity of the method. MAb 143.9 as well as 143.10 neutralise the virus and recognise an epitope located at the N-terminal part of VP1. Seven amino acid differences are found between ITL/1/66 and NET/1/92, in that region (Figure 3). Amino acid 87 of VP1 has previously been identified to be important in neutralisation site 1 (Kanno et al., 1995). MAbs 143.9 and 143.10 might recognise the same site. In the C-terminal part of VP1 we find five amino acid differences, of which one is located on the GH loop and three are located on the last 25 amino acids. In foot-and-mouth disease the GH loop contains very important epitopes and is probably the site that is recognised by the cell receptor (Crowther et al., 1993). No epitopes have been located on this loop in SVDV, therefore the last 25 amino acids, which contain neutralisation site 3a (Nijhar et al., 1999), are a good candidate for the localisation of the epitopes of neutralising MAbs, 145.2 and 145.12. The epitopes of non-neutralising MAbs, 143.1, 143.2 and 143.3, however, might be located on a different location. Production of single site and multiple site directed mutants will be described in a subsequent paper, which will give more information on the exact location of the epitopes recognised by the MAbs. The information presented here shows that production of chimeric viruses by fusion PCR can be used very easily to map epitope regions of particularly non-neutralising MAbs on picornaviruses. But this method can only be applied if the MAbs of interest can discriminate between two isolates.

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Chapter 5

Validation of a monoclonal antibody-based ELISA to detect antibodies directed against swine vesicular disease virus

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Abstract

A simple, rapid and sensitive competitive monoclonal antibody-based ELISA for the detection of antibodies directed against swine vesicular disease virus (SVDV) was developed. The ELISA was validated using field sera originating from SVDV-infected and non-infected Dutch pig herds, reference sera obtained from the Community Reference Laboratory for Swine Vesicular Disease at the Institute for Animal Health, Pirbright Laboratory, U.K., and sera from experimentally infected animals. When testing 4277 sera originating from non-infected Dutch pig herds and collected as part of the national screening program, this ELISA had only 0.6 % false positive results, whereas approximately 2 % was obtained with a conventional blocking ELISA used until recently. A sensitivity relative to the virus neutralisation test of >97% was achieved when testing sera collected from Dutch pig farms where an outbreak of SVDV had occurred. All international reference sera scored consistently correct. Sera sequentially collected from pigs experimentally infected with SVDV isolates representing all currently recognized antigenic groups, were scored positive slightly earlier in the ELISA compared to the virus neutralisation test. This monoclonal antibody-based competion ELISA for SVDV antibodies designated the Ceditest ELISA for SVDV-Ab, is as sensitive but more specific than the ELISA used until recently. Because sera are tested in a single dilution (1:5), incubations are performed at room temperature and test results are available within 3 hours, this ELISA is simple, easy to automate and therefore highly suitable for screening large numbers of serum samples.

1 Introduction

Swine Vesicular Disease (SVD) is a highly contagious disease of pigs caused by an enterovirus. Infected pigs may develop vesicles and fever (Kitching, 1995). SVD is important because it is clinically indistinguishable from Foot-and-Mouth-Disease (Nardelli et al., 1968; Kitching, 1995). For this reason, outbreaks of vesicular disease must be assumed to be Foot-and-Mouth-Disease until diagnosis proves otherwise. SVD is a notifiable disease in the European Union (EU) and warrants controls such as movement restrictions and slaughter in the event of an outbreak. An outbreak of SVD therefore demands swift, effective response to limit the spread of the disease. The spreading of SVD can be controlled by implementing movement restrictions and rapidly screening contact pig herds. Epidemiological surveillance is essential in order to maintain a disease-free status. Although the virus neutralisation test (VNT) is accepted as the gold standard, large scale screening of pig herds for SVD is most efficiently done by ELISA. The need for an efficient ELISA becomes obvious when considering the large numbers of sera tested in large scale screening programs. Since March 1993, the Netherlands has been screening its pig herds for the presence of antibodies directed against SVD virus as part of the national surveillance program (Terpstra et al., 1993). In 1994-1996 approximately 2,250,000 sera have been tested in the Liquid Phase Blocking ELISA (Dekker et

al., 1995). Although this test is highly sensitive, it is more laborious and more time consuming than the test currently described. Since 2% of the results have been false positive (test specificity is approx. 98%), about 15,000 samples had to be submitted each year for confirmation in the VNT. Although 2% false positive results is normally considered acceptable, when large numbers of samples have to be tested, it is not.

Therefore, we developed an ELISA based on the Monoclonal Antibody-based Competitive ELISA (MAC-ELISA) described by Brocchi et al. (1995) and introduced a technical production innovation which makes a much shorter test procedure possible. The total time to perform the test has been shortened to less than 3 hours.

The study presented here, describes the validation of this test, designated Ceditest ELISA for SVDV-Ab, using field sera from SVDV infected and non-infected Dutch pig herds, reference sera distributed by the Community Reference Laboratory for Swine Vesicular Disease at the Insitute for Animal Health, Pirbright Laboratory, U.K., (CRL) and sera from experimentally infected animals.

2 Materials and methods

2.1 monoclonal antibodies

Anti-SVDV MAb 124-11-A (mouse, isotype IgG2a) was used for the coating as well as for the conjugate. It was selected from a panel of monoclonal antibodies raised against SVDV strain UKG/27/72 (Dekker, personal communication, 1996). The MAb was purified from cell culture supernatant and either stored at -20 □C in 50 % (v/v) glycerol, or conjugated to horseradish peroxidase using the method described by Wilson and Nakane (1978), and stored at 4□C with conjugate stabilizer. A second and irrelevant MAb (mouse, isotype IgG2a) was grown in cell culture, aliquoted, freeze dried, stored at 4□C and used as an additive to the conjugate buffer.

2.2 Sera

Three sets of field sera were used for validation of the test. The first set consisted of sera from non-infected pig herds (n = 4277) collected recently as part of the national surveillance program, the second and third set of sera are from a SVD virus infected breeding herd (n = 263) and sera from a SVD virus infected fattening herd (n = 354) as described by Dekker et al. (1995).

The CRL supplied the reference sera in the form of the 1995 SVD reference panel (n=6). This panel included the EU standard low positive serum RS 01.04.93, which is defined by as being the lowest level of SVD antibodies that should consistently score positive in the ELISA as well as in the VNT (Mackay et al., 1996).

Sera (n=320) from experimental infections were sequentially collected from specific pathogen free pigs following intradermal infection with one of the following SVD virus isolates: ITL/1/66, BUL/2/71, UKG/27/72, HKN/11/81,

ITL/2/91, NET/1/92, NET/2/92. These isolates represent all current SVD virus groups characterised by antigenic and molecular biological methods (Brocchi et al., 1997).

2.3 Virus neutralisation test

The VNT was performed as described by Dekker et al. (1995). Serum titres higher than 2.0^{10}log were considered positive.

2.4 ELISA

The principle of the Ceditest ELISA for SVDV-Ab is similar to the MAC-ELISA described by Brocchi et al. (1995). ELISA plates were coated with MAb 124-11-A by a 2 h incubation at room temperature, washed, coated with 1% (w/v) Bovine Serum Albumin by a 30 minute incubation at room temperature, emptied without washing, coated with inactivated SVD antigen (UKG/27/72) by an overnight incubation at 4℃, washed, dried, sealed in plastic bags with separately packaged silica gel and stored at 4℃. The ready-to-use stable test plates, containing captured antigen, allowed results to be obtained after a short, simple test procedure.

Before use, the test plates were equilibrated at room temperature. Volumes of 50 µl of control serum or test serum diluted 1:5 and 50 µl of diluted conjugate (containing 0.04 mg/ml irrelevant mouse IgG2a) were added to each well. The plates were sealed and incubated for 2 h at room temperature. The test plates were then washed whereafter 100 µl of ready-to-use chromogen substrate solution (2-4-6-6 tetra methyl benzidine/peroxide) was added to each well. After 20 minutes at room temperature, the colour development was stopped using 100 µl of 0.5 M H2SO4 and the optical density at 450 nm was measured. Sera were considered positive (specific antibodies present) if the colour development, after subtraction of a blank, was inhibited for more than 50% when compared to a standard negative reference serum. Four reference sera were tested in duplicate on every test plate and served to check the validity of the test as outlined by Kramps and Van Rooij (1997). Reference serum 1 was strong positive and served as the blank, reference serum 2 was weak positive, reference serum 3 (diluted positive serum) contained specific antibodies below the detection level of the test, and reference serum 4 was a standard negative serum. Percentage inhibition (% Inh) of test and reference sera were calculated using the following formula:

$$\%\ inhibition = 100 - \left(\frac{OD\ test\ serum - mean\ OD\ ref.\ serum\ 1}{Mean\ OD\ ref.\ serum\ 4 - mean\ OD\ ref.\ serum\ 1} \right) \times 100\%$$

Washings were performed with demineralized water containing 0.05% Tween 80.

2.4 Mathmatics

Sensitivity and specificity were determined according to Diamond (1989), using the VNT as the gold standard. Sensitivity is defined as the probability that a serum containing SVDV specific antibodies will be diagnosed as positive. Specificity is defined as the probability that a serum without SVDV specific antibodies will be diagnosed as negative. The formulas used were:

$$Sensitivity = \left(\frac{\text{Number of true positives with a positive result}}{\text{Number of all true positives}} \right) \times 100\%$$

$$Specificity = \left(\frac{\text{Number of true negatives with a positive result}}{\text{Nmber of all true negatives}} \right) \times 100\%$$

"True positive" = VNT titre $\geq 2.0\ ^{10}\log$
"True negative" = VNT titre $< 2.0\ ^{10}\log$

Due to the fact that the VNT generates some false positive results, (Brocchi et al., 1995, Dekker et al., 1995) the term "True Negative" was also applicable when a VNT positive pig had no signs of clinical disease and the herd had no history of disease, and when only 1 pig per herd had a positive result in the VNT. These pigs are called singleton reactors (Mackay et al., 1995).

Reproducibility was assessed by analysing the results of a large number sera tested on two separate occasions. Reproducibility is a measure of the degree to which two independent observations agree with each other and can be quantified in different ways. In this validation, a kappa coefficient (KD) and a total agreement were calculated (Feinstein and Cicchetti, 1990).

The threshold or cut-off value above which a sample was considered positive was determined by analysing the sensitivity and specificity of the ELISA over a range of cut-off values as described by Stegeman et al. (1996)

3 Results

To determine the appropriate cut-off value, 4894 sera originating from Dutch pig herds were tested in the Ceditest ELISA for SVDV-Ab. Of these, 4277 originated from herds free of clinical signs of SVD and 617 sera (of which 250 were positive in the VNT) came from two SVD infected herds.

The frequency distribution (Fig. 1) of the percentage inhibition shows that 4252 of the 4277 (99.4 ± 0.1%) sera originating from non-infected herds have less than 50% inhibition and 244 of the 250 (97.6 ± 1.0%) VNT positive sera, originating from infected herds, have greater than 50% inhibition. Of the 25 sera originating from healthy herds that scored more than 50 % inhibition in the

ELISA, three were scored positive by VNT with (^{10}log) titres of 2.0 to 2.3, ten sera had VNT titres between 1.7 and 1.9 and twelve sera had titres less than 1.7. The set of 4277 sera were also tested at the Regional Animal Health Service in Boxtel (province of Brabant, NL) using the Liquid Phase Blocking ELISA (Dekker et al., 1995) where 2.6% scored positive. Sensitivity and specificity and the accompanying 95% confidence intervals (CI) were calculated for all cut-off values from 0 to 100% at intervals of 5% (data not shown). The optimally efficient (maximises both sensitivity and specificity) cut-off value was determined to be 50%. At this cut-off value, a sensitivity of 97.6% (CI: 95%-99%), and a specificity of 99.4% (CI: 99%-100%) were found.

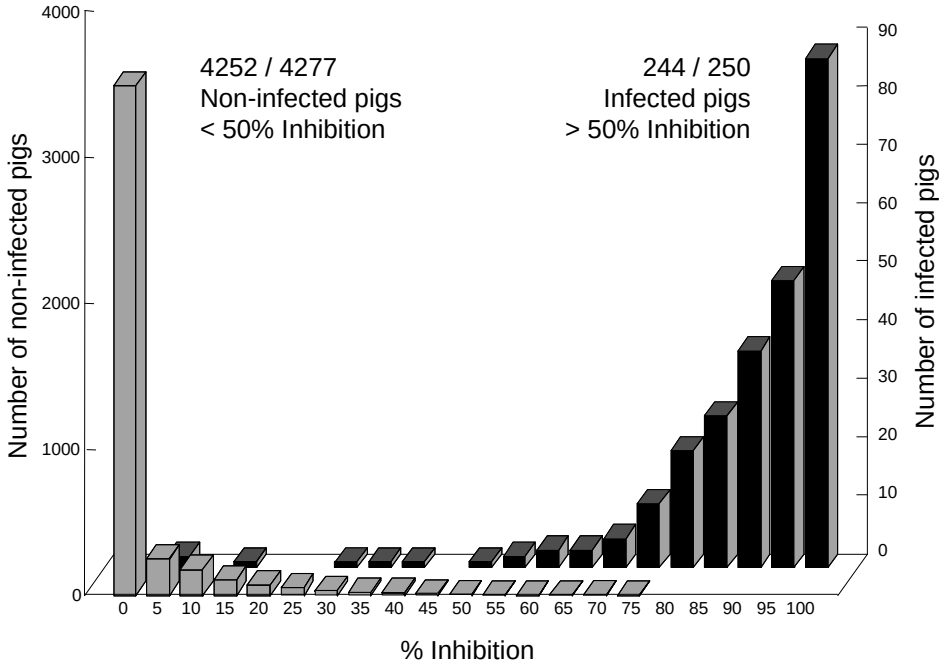

Figure 1: Frequency distribution of percentage inhibition of sera (n = 4277) collected from Dutch pig herds free of SVD and of sera (n = 250) collected from Dutch pig herds infected with SVD.

Testing the EU low positive standard serum (RS 01.04.93) resulted in a mean % inhibition of 67 ± 6.9% (range 53 - 79%) when tested 95 times on 11 different days. The five remaining sera of the 1995 reference panel distributed by the CRL were also correctly scored (data not shown).

Table 1: Number of pigs positive out of total in the ELISA and VNT for different virus isolates at days post infection

Virus isolate	Test	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	20	21	22 to 49[1]
ITL/1/66	ELISA	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/2	1/2	1/2	2/2	2/2	-	2/2	2/2	-	3/7
	VNT	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/2	1/2	-	1/2	1/2	-	2/7
BUL/2/71	ELISA	0/2	0/2	0/2	2/2	2/2	-	2/2	-	-	2/2	-	2/2	-	-	-	-	2/2	4/4
	VNT	0/2	0/2	0/2	0/2	2/2	-	2/2	-	-	2/2	-	2/2	-	-	-	-	2/2	4/4
UKG/27/72	ELISA	-	-	-	-	5/5	-	-	-	5/5	-	-	5/5	-	-	5/5	-	5/5	17/17
	VNT	-	-	-	-	5/5	-	-	-	5/5	-	-	5/5	-	-	5/5	-	5/5	17/17
HKN/11/81	ELISA	0/2	1/2	2/2	2/2	2/2	-	2/2	-	-	2/2	-	2/2	-	-	-	-	2/2	4/4
	VNT	0/2	1/2	2/2	2/2	2/2	-	2/2	-	-	2/2	-	2/2	-	-	-	-	2/2	4/4
ITL/2/91	ELISA	0/2	2/2	2/2	2/2	2/2	-	2/2	-	2/2	-	-	2/2	-	-	-	-	2/2	18/18
	VNT	0/2	0/2	2/2	2/2	2/2	-	2/2	-	2/2	-	-	2/2	-	-	-	-	2/2	18/18
NET/1/92	ELISA	-	0/5	-	0/5	-	0/5	-	-	0/5	-	-	0/5	-	-	0/5	-	0/5	19/34
	VNT	-	0/5	-	0/5	-	0/5	-	-	0/5	-	-	0/5	-	-	0\5	-	0/5	19/34
NET/2/92	ELISA	0/4	-	-	-	1/4	-	-	4/4	-	-	4/4	-	-	4/4	-	4/4	-	24/26
	VNT	0/4	-	-	-	0/4	-	-	4/4	-	-	4/4	-	-	4/4	-	4/4	-	16/26
NET/2/92	ELISA	-	0/5	3/5	4/5	-	4/4	-	-	5/5	-	3/3	-	5/5	-	-	-	5/5	5/5
	VNT	-	0\5	1/5	3/5	-	4/4	-	-	5/5	-	3/3	-	5/5	-	-	-	5/5	5/5
Total	ELISA	0/12	3/18	7/13	10/18	12/17	4/11	6/8	4/6	13/19	5/6	8/9	13/18	7/7	4/4	7/12	6/6	16/21	94/115
	VNT	0/12	1/18	5/13	7/18	11/17	4/11	6/8	4/6	12/19	4/6	7/9	12/18	6/7	4/4	6/12	5/6	16/21	85/115

[1] The duration of the infection experiments varied from 4 to 7 weeks. This column is an enumeration.

Sera collected from specific pathogen free pigs following experimental infection scored positive in the ELISA on the same day, or in most cases, one or more days earlier compared to the VNT (Table 1). This was observed with sera raised against every SVD virus isolate tested. Of the 320 sera tested, 195 scored positive in the VNT, all of which were scored positive in the ELISA along with 24 VNT negative sera. The ability of the ELISA to detect antibodies after infection was equal to, or better than the ability of the VNT when testing experimental sera.

Sera collected from infected herds and sera collected following experimental infection (n = 937) were tested on separate occasions using ELISA test plate batches from different production runs. The reproducibility of the Ceditest ELISA for SVDV-Ab is high, a K_D of 0.94 and a total agreement of 97% ((493⊕ + 414⊖) / 937 x 100%) were obtained. Thirty sera had a different result in the second test. Of these, only three were VNT positive (originating from an SVDV-infected herd).

4 Discussion

The aim of this study was to validate this monoclonal antibody-based competition ELISA for the detection of antibodies directed against SVDV. This ELISA was designed to meet important diagnostic requirements for use in large-scale monitoring and eradication programs. The test has important advantages over the VNT and other available ELISAs for the detection of SVDV antibodies. The VNT requires 4 days for completion and is not suitable for automation. Advantages over the MAC-ELISA (Brocchi et al., 1995) are mainly logistic: test plates (as well as all other necessary ingredients) are ready-to-use and stable for a long period of time. Results with the Ceditest ELISA test kit can be obtained within 3 hours after only 1 wash step and 2 incubation steps at room temperature.

As stated previously, 244 of 250 (97.6 ± 1.0%) VNT positive field sera collected from infected Dutch pig herds correctly scored positive in the Ceditest ELISA. The six VNT positive field sera, which scored negative in the ELISA in the screening dilution of 1:5, all scored positive in the ELISA at dilutions ranging from 1:20 to 1:80. Increasing the concentration of the irrelevant IgG2a had no effect on the number of false negative results whereas an absence of the irrelevant IgG2a resulted in a two to three-fold increase in false negative results (data not shown). These false negative results are likely due to the binding of the peroxidase labelled MAb to the capture MAb by proteins present in pig serum. These proteins are probably heterophilic antibodies that have a broad binding capacity including an affinity to mouse IgG as suggested by Levinson (1992). The sensitivity of 97.6 ± 1% is the sensitivity for individual animals and is different from herd sensitivity. Herd sensitivity depends on test sensitivity, sample size and the expected prevalence of the infection. Therefore missing some individuals can be compensated for by increasing the sample size (Dekker

and Terpstra, 1996). This minimises any drawbacks associated with testing in a single dilution.

The ability to correctly diagnose SVD antibody negative pigs was assessed using sera from non-infected Dutch pig herds. Only 25 of 4277 sera (0.6%) gave a positive result. Approximately half of these 25 sera had titres below or slightly above the cut-off in the VNT. The cause of this reactivity is unlikely to be SVD since the animals showed no clinical signs of disease and only one pig per herd was positive in the VNT. The sera therefore should be considered false positive and the animals can be classified as singleton reactors according to EU guidelines. Since early 1997, more than 500,000 sera originating from Dutch pig herds free of clinical signs of SVD have been tested in the Ceditest ELISA for SVDV-Ab. Approximately 1% of the results have been false positive. The fact that both the VNT and the ELISA generate some false positive results agrees with earlier findings (Brocchi et al., 1995, Dekker et al., 1995).

When testing the reference sera and sera from experimentally infected pigs, all of the VNT-positive sera were correctly scored as positive, satisfying the criterium set by the EU. The SVD virus isolates used for experimental infection represent all currently recognised antigenic groups. Seroconversion times varied from 4 to 22 days post infection which may be explained by differences in the virulence of the strains used. This would agree with earlier observations that some SVD virus strains produce only very mild or no clinical disease in some pigs (Burrows et al., 1974, Donaldson et al., 1983, Brocchi et al., 1995). Although earlier studies (Dekker, personal communication, 1996) showed that MAb 124-11-A does not recognize SVDV isolates ITL/1/66, HKG/11/81 nor the Dutch 1992 isolates, antibodies directed against these isolates were detected in the ELISA. This indicates that conjugated MAb 124-11-A is sufficiently blocked by antibodies that do not compete for the same epitope suggesting that antibodies directed against SVDV isolates ITL/1/66, HKG/11/81 and the Dutch 1992 isolates are able to block conjugated MAb 124-11-A due to steric hindrance. This strengthens the possibility that antibodies directed against future antigenic variants of SVD virus will be detected with the Ceditest ELISA for SVDV-Ab.

Our results show that the Ceditest ELISA for SVDV-Ab is sensitive, specific and highly suitable for use in monitoring and eradication programs where large numbers of sera have to be tested. The sensitivity was determined to be >97 % relative to the VNT when using well defined positive sera originating from infected Dutch pig herds or the EU low positive reference serum (RS 01.04.93) which scored consistently positive. The specificity was determined to be greater than 99 % when testing non-infected pigs. Therefore, this ELISA has the ability to correctly diagnose a seropositive pig while keeping false positive results to a minimum. Additionally, the high reproducibility (KD 0.94, total agreement 97%) emphasises that the Ceditest ELISA for SVDV-Ab is a reliable diagnostic tool. Because sera can be tested in a single dilution and incubated at room temperature, this test is easy to perform and easy to automate. Furthermore, the use of *in vitro* produced monoclonal antibodies eliminates the need for ascitic fluid. This

ELISA is evidently highly suitable for herd testing and can therefore be adopted as a standard ELISA for monitoring and eradication programs in support of international trade.

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Chapter 6

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

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Abstract

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 day 3 to 49 and occasionally up to day 91 after infection. IgG_1 antibodies were first detected at day 8 and IgG_2 at day 11. IgA antibodies coincided with IgG_1 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_{50}). A D_{50} of 5, 4, 12, 12 and 24 days was calculated, respectively, for the appearance of antibodies in the virus neutralisation test, the IgM, total IgG, IgG_1 and IgG_2 ELISA. A D_{50} 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.

1 Introduction

Swine vesicular disease (SVD) is an infectious disease of pigs first recognised in 1966 in Italy (Nardelli et al., 1968). As the clinical signs resemble those of foot-and-mouth disease, SVD is classified as an OIE list A disease. SVD control in Europe is solely based on stamping out of infected herds. In case of an outbreak, it is essential to trace contacts leading to the source of the infection, and possible to secondary outbreaks. For efficient tracing, information on the epidemiology of the disease is essential. The main entry site of the virus leading to clinical disease, is the skin (Burrows et al., 1974; Mann and Hutchings, 1980; Dekker et al., 1995b). Inoculation on that predilection site, however, even with high doses, does not always produce clinical signs in all inoculated pigs (Burrows et al., 1974). We showed previously that infection via indirect contact, which simulates the field situation, was more effective than direct inoculation of animals (Dekker et al., 1995b). The epidemiology of a disease cannot be studied in an experimental setting alone. Field observations together with laboratory studies are essential to understand the epidemiology, because in field observations also population dynamics are taken into account. Field epidemiological data are scarce, because 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 (Hedger and Mann, 1989). The latter was probably the route of introduction into Great Britain. Mathematical models are helpful to gain insight into the population dynamics of infectious diseases (De Jong, 1995). Without reliable epidemiological data, however, it is not

possible to develop a valid model. More information is therefore needed on the routes by which SVD virus (SVDV) is spread within and between herds.

Nine outbreaks of SVD have occurred in the Netherlands in 1992 and 1994. The spread of the disease could be traced to direct contact with diseased animals in three cases only. The route of transmission in the other outbreaks was most probably due to contact with contaminated transport vehicles, although other routes could not be excluded. To understand infection dynamics within and between herds, it is essential to estimate the time of infection. For this purpose we developed and evaluated five isotype specific ELISAs to detect antibodies against SVDV. Virus specific IgM and IgG ELISAs have been described previously (Brocchi et al., 1995), but we also included IgG1, IgG2 and IgA ELISAs.

The newly developed isotype specific ELISAs were validated with sera from four SVD infection experiments, and were used to estimate the time of virus introduction on all Dutch outbreak farms diagnosed in 1992 and 1994.

2 Materials and methods

2.1 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).

2.2 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 $TCID_{50}$ per pig) of SVDV into the bulb of the heel and housed in high security stables of the Institute.

2.3 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 neutralisation 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_1 and IgG_2 ELISAs

2.2.1 1992 outbreaks

Outbreak 92-01, 92-02, and 92-03 were diagnosed by antigen detection (Hamblin et al., 1984) 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 one kilometre from each other. Table 1 shows the type of farm, herd size and the sampling procedure followed for each outbreak farm.

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

Outbreak	Date of detection	Type of farm[1]	Herd size				Sampling procedure
			sows	boars	piglets	Fatteners	
92-01	3-7-92	B	102	3	488	0	Selective
92-02	13-7-92	B	92	1	397	11	All sows, some piglets
92-03	30-7-92	F	0	0	0	352	Two pigs per pen
92-04	29-9-92	B+F	264	2	±1000	20	All sows, and some piglets and fatteners
92-05	29-9-92	C	0	0	0	9	All pigs
92-06	27-10-92	F	0	0	0	365	All pigs
94-01	15-2-94	C	0	0	0	0	Faeces, randomly
94-02	18-2-94	F	0	0	0	22	All pigs
94-03	24-2-94	C	0	0	0	0.	Faeces, randomly

[1] B = breeding farm
F = fattening farm
C = Collection centre

2.3.2 1994 outbreaks

At the export collection centre (94-01), faecal samples were taken after SVD had been diagnosed in Italy in pigs 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 the epidemiological link. On farm 94-02, serum samples were taken from all 21 fattening pigs, of which 17 were positive. On 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.

2.4 Monoclonal antibodies

Monoclonal antibodies (MAbs) specific for porcine IgM (CVI.28.4.1), IgG (CVI.23.3.1a), IgG_1 (CVI.23.49.1), IgG_2 (CVI.34.1.1a) and IgA (CVI27.9.1b) have been described earlier (Van Zaane and Hulst, 1987). SVD virus specific MAbs, CVI.124.8 and CVI.124.11 were produced against SVD virus isolate UKG/27/72 (Dekker et al., 1999). 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_1 and IgG_2 specific MAbs were conjugated with horseradish peroxidase as described by Wilson and Nakane (1978).

2.5 Serological tests

2.5.1 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% foetal 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 orthophenylenediamine 0.015% H_2O_2) for 15 min at room temperature, stationary and in the dark. After 15 min, the colour reaction was stopped by the addition of 100 µl 1M H_2SO_4 to each well.

2.5.2 IgG, IgG_1 and IgG_2 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_1 or IgG_2, respectively. After a final washing 100 µl of chromogen-substrate solution was added to each well. Colour development was allowed for 15 minutes, stationary in the dark, and was stopped by the addition of 100 µl 1M H_2SO_4 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 ^{10}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 6 times with tap water containing 0.05% tween 80.

2.5.3 Direct liquid phase blocking ELISA

The direct liquid phase blocking ELISA was performed as previously described (Dekker et al., 1995a).

2.5.4 Virus neutralisation test

The virus neutralisation test was performed as described earlier (Dekker et al., 1995b). Sera with a neutralisation titre equal or above the European standard serum (RS 01.04.93) were considered positive (Mackay et al., 1996).

2.6 Virus isolation

Virus isolation was performed on monolayers of IBRS-2 cells (de Castro, 1964) as described previously (Dekker et al., 1995b)

3 Results

3.1 Experimental infections

All inoculated pigs showed clinical signs characteristic for SVDV. The severity of the lesions, however, differed between pigs receiving different strains, especially strain BUL/2/71 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 (Figure 1, Table 2). Individual pigs scored positive in the IgM ELISA 0 to 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_1 and IgG_2 ELISA are shown in Figure 2. The IgG_1 response was comparable to the total IgG response, starting at the same time and reaching approximately

the same titres. The height of the IgG_2 response was lower than the total IgG response, and started approximately 2 weeks later (Figure 2). 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). After 3 to maximally 6 days post infection (DPI), the first period started in which only the IgM ELISA scored positive. In the infection experiments, this first period lasted from 3 to 7 days. In individual pigs, however, this period lasted maximally two days, but could also be absent. This first period was followed by a second period, between 4 and 15 days after infection, 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 DPI, pigs tested positive for IgM, IgG and IgG_1 antibodies. Starting from day 11, IgG_2 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 days after infection, but after 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 in each test. The D_{50} was 5 days for the VNT, 4 days for IgM ELISA and 12, 12 and 24 days for the appearance of total IgG, IgG_1 and IgG_2 ELISA, respectively. A D_{50} of 49 days was calculated for the disappearance of IgM antibodies (Table 2).

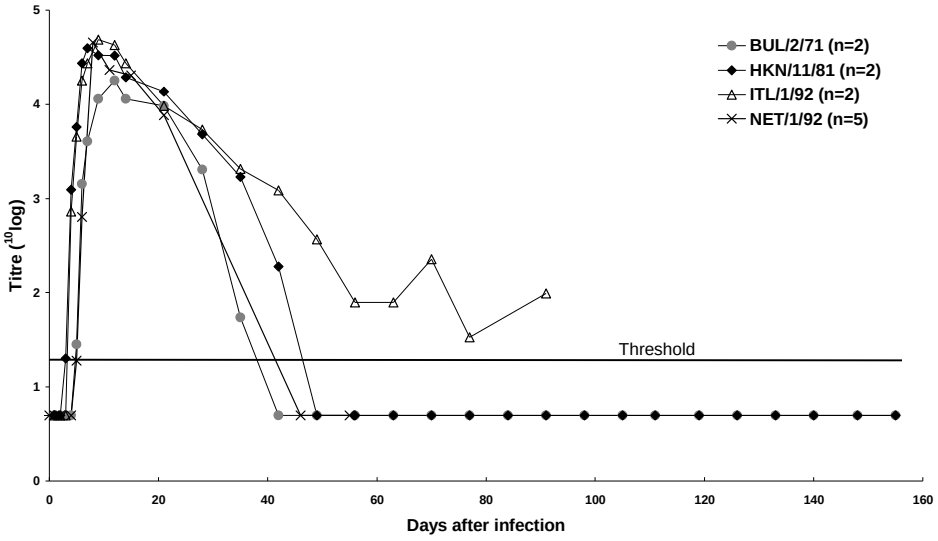

Figure 1: Average IgM antibody response after experimental SVDV infection with SVDV isolates BUL/2/71, HKN/11/81, ITL/1/92 and NET/1/92.

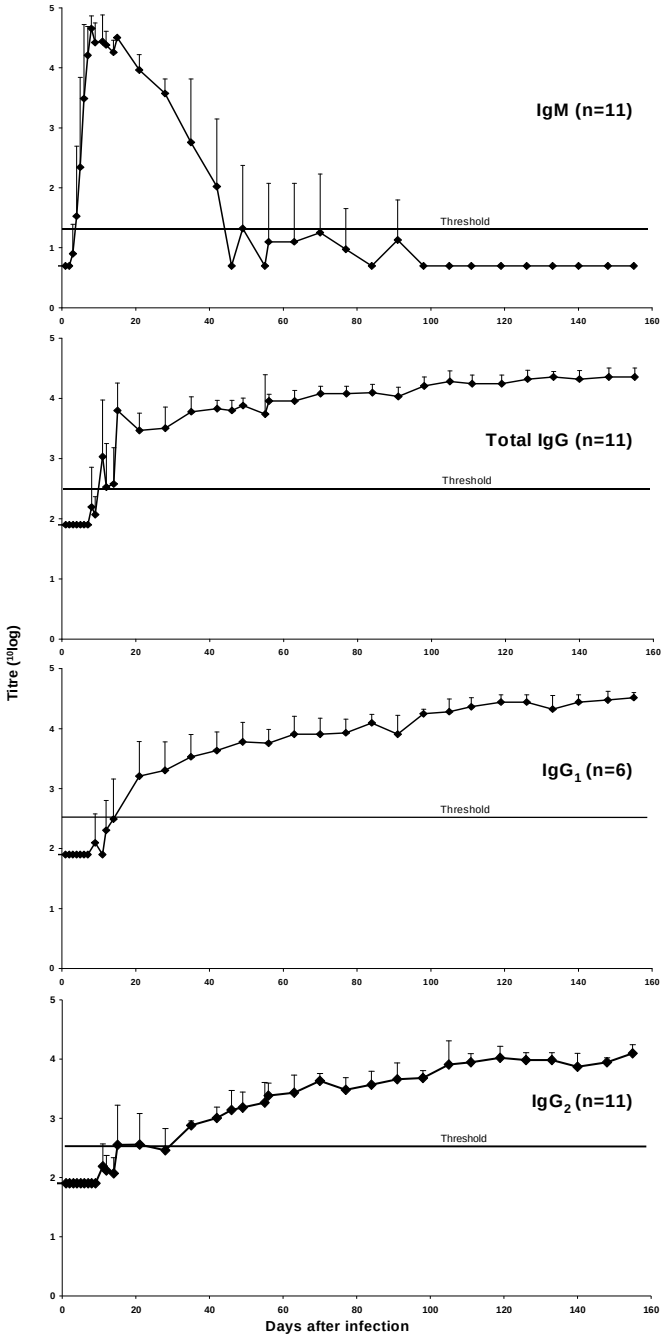

Figure 2: Average IgM, IgG, IgG1 and IgG2 responses (bars represent the standard deviation) after experimental infection with various SVDV isolates.

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

Test	Days after infection (based on all observations)	D_{50}[1]	95% confidence interval D_{50}
Start			
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_1	8 - 28	12	10.7 - 14.1
IgG_2	11 - 35	24	20.3 - 28.4
End			
IgM	35 - 91	49	42.8 - 55.3

[1] D_{50} = The day that 50% of the animals became positive or negative (IgM) in the test.

3.2 Field sera

To check the specificity, 156 VNT-negative field sera were tested in the IgM, IgG, IgG_1 and IgG_2 ELISA, resulting in 4, 3, 15 and 11 positive samples, respectively. Therefore, the specificity of the IgM, IgG, IgG_1 and IgG_2 was 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 not longer available so only positive sera could be tested. On farm 92-03 25 out 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 out of 266) was found on farm 92-04. On this farm nine serum samples with low VNT titres (2.30 and 2.45), were negative in the IgM and IgG ELISA. These nine sera were positive in the liquid phase blocking ELISA (Dekker et al. 1995a), and 8 out of 9 scored positive in the IgG_1 or IgG_2 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_2 ELISA, 153 out of 266 being positive. Fourteen of the 153 positive sera were negative in the IgG ELISA; seven of these 14 were negative in the VNT, and one contained IgM.

On farm 92-02, 92-03, 92-04 and 92-06, the results of all tests were analysed as shown in figure 3. On farm 92-02, 92-03 and 92-06, the positive pigs were clustered. On farm 92-02, 7 out 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 located on the other side of the same section. The results of the tests and the location of the seropositive pigs on farm 92-03 are shown in Figure 3. 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 localised 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. In the first section, 8 out of 19 pigs were positive with only IgG antibodies, in the second section only one serum was positive with only IgG antibodies. Nine seropositive pigs, housed in two pens, had entered this farm 16 days prior to sampling. All nine pigs were positive in the IgM ELISA, six were also positive in the IgG ELISA. On farm 92-04, the seropositive pigs were more or less randomly distributed on the farm. Pigs with only IgM antibodies were found in six of the 10 sections, pigs with IgM and IgG antibodies were found in four of the 10 sections. In addition, pigs with only IgG antibodies were found in six of the10 sections.

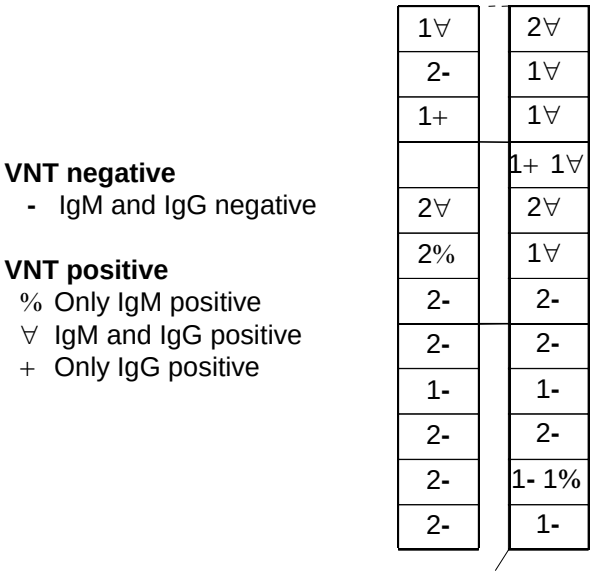

Figure 3: Distribution of the results of the VNT, IgM and IgG ELISA on serum samples collected in 21 pens of one stable of outbreak farm 92-03. In two other stables, all 19 samples taken from 13 pens were negative in all tests.

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

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

N.T. = Not tested

4 Discussion

The aim of this study was to develop isotype specific ELISAs to detect antibodies against SVDV and that can be used to estimate the time an SVD outbreak started. Five isotype specific ELISAs were developed, and validated on negative field sera and sera from experimentally infected animals. We used animals infected in the bulbus of the heel, although this is probably not the natural infection route. Previous experiments had shown that animals in contact with a contaminated environment become infected the first day of contact, and neutralising antibody titres were comparable to those found in animals infected in the bulbus of the heel (Dekker et al., 1995b). The day IgM was first detected was comparable to that found by Brocchi et al. (1995). However, we were able to detect IgM antibodies beyond 25 days after infection, which was the limit of detection in the previously described test. In contrast to the indirect IgM ELISA used by Brocchi et al. (1995) we used an antibody capture assay (ACA) for detection of both IgM and IgA. This approach has among others been used for bovine rotavirus (van Zaane and IJzerman, 1984), bovine respiratory syncytial virus (Kimman et al., 1987), pseudorabies virus (Kimman et al., 1992) and influenza virus (Heinen et al., 1999). Using this ACA format for IgM and IgA has helped to overcome antibody competition between IgM or IgA, and IgG antibodies (van Zaane and IJzerman, 1984). The design of the IgG, IgG_1 and IgG_2 ELISAs used in this study was comparable to the IgG ELISA described by Brocchi et al. (1995). Responses of total IgG, IgG_1 and IgG_2 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 two animals were still IgM positive 91 days after

infection makes it difficult to determine the exact endpoint of the IgM response. No explanation could be found for the differences in IgM response between the different infection experiments (Figure 1). Because of the 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 35 days ago (Table 2). Because farm 92-04 was suspected of having been infected for more than three months, we looked for a method to estimate the time of infection beyond this period. In some pigs the SVD specific IgA antibody response seemed to last for approximately 150 days, but variation in IgA responses between pigs was considerable. The results obtained on a few outbreak farms confirmed this variation. So the IgA response could not be used to estimate the time that elapsed after infection. The long lasting IgA response, however, was remarkable. Because IgA is specific for mucosal surfaces the differences between pigs might be caused by differences in the involvement of the intestinal mucosa. After oral (Lin et al., 1998) and intradermal infection into the bulb of the heel (Burrows et al., 1974) SVDV can be found in faecal samples. After oral infection some pigs excreted virus in their faeces for 126 days (Lin et al., 1998), in contrast to the intradermally infected animals where virus could not be isolated after the 7th day (Burrows et al. 1974). Perhaps the duration of the intestinal infection is correlated with the height and length of the IgA response.

Based on the negative field sera the specificity of the IgM and IgG ELISAs was over 95%. The specificity of the IgG_1 and IgG_2 ELISAs was somewhat lower. Based on the results 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.

On the two farms detected because of clinical suspicion by the owner (92-01 and 92-02), we found the highest proportion of IgM positive sera (Table 3). On farm 92-01, blood samples were selectively taken from pigs with clinical symptoms, which almost certainly has caused a bias toward IgM antibodies. Two pigs with IgM and IgG and without IgG_2 antibodies were found on this farm, indicating that the herd, based on D_{50}, had been infected between 12 and 24 days earlier. This is consistent with the observation of the farmer who had seen udder lesions two weeks earlier. On farm 92-02, one animal showed a positive IgG response, indicating that this herd was infected at least 8 days previously. So this farm was already infected before the pigs of the neighbouring outbreak (92-01), less than 30 meters away, were culled. This affirms the view that farm 92-02 was probably infected by contact with farm 92-01. The nature of the contact could not be determined, but was unlikely due to the movement of pigs.

Based on the D_{50} value farm 92-03 must have been infected at least 49 days before detection, because two VNT-positive pigs without IgM antibodies were found (Table 3). A total of 17 of the 19 IgG positive sera also contained IgG_2, indicating that the infection had spread to those pens between 24 and 49

days before sampling. Contact between the pens in the sections shown in Figure 3 did not result in infection of all the pens in those two sections. This confirms the observation of Hedger and Mann (1989) that SVD is a "pen disease" rather than a "farm disease". Despite the use of the isotype specific ELISAs, it is still not possible to determine whether farm 92-03 was infected before or after farm 92-01 became infected, the infection dates were probably very close. Tracing, however, did not reveal a common source.

The estimation of the date of virus introduction on farm 92-04 was very interesting from an epidemiological point of view. The fact that 76% of the VNT-positive sera were negative for IgM indicates that those pigs must have been infected at least 49 days before sampling, a finding supported by udder lesions observed 2 months earlier. A better estimation of the time of infection based on serology is, however, not possible. In contrast to the other outbreaks, the positive pigs were not restricted to one part of the farm. Pigs with only IgM antibodies, thus recently infected, were found in several sections, indicating that the infection was actively spreading on the farm. The spread of the infection in this farm was probably caused by movement of pigs within the farm, which is normal practice in breeding herds. None of the farms that had received piglets from farm 92-04 became infected, neither were these piglets serologically positive. Unfortunately, only a few piglets on farm 92-04 were tested; the older ones on the flatdeck were negative, but four weaned piglets in the farrowing stable were positive in the VNT and IgM ELISA, but not in the IgG ELISA. Knowing that this farm must haven been infected for more than two months, and probably longer, it is incredible that no virus had been spread by selling fattening pigs. The sows were probably infected a long time before farrowing and not infective anymore at the time they gave birth. Lin et al. (1998) found in one infection experiment that all faecal samples became positive again for SVDV genome 121 days after infection, after two groups of three infected pigs were stressed by mixing. These authors suggested that the pigs, which started excreting virus 121 days after infection, might be the cause of new infections. In farm 92-04, almost none of the sows spread the virus to their offspring. This indicates that the spread suggested by them cannot be of great epidemiological significance.

The sera from outbreak 92-04, revealed seven VNT-negative, total IgG negative but IgG_2 positive sera. These sera may be considered false positive in the IgG_2 ELISA; this finding is consistent with the test specificity of 93%. Nine sera were found positive by VNT and eight of them positive in the IgG_1 and IgG_2 ELISA, but negative in the IgG ELISA. The antibody titres were probably low, and therefore may have resulted in a false negative result in the total IgG ELISA.

The export collection centre 92-05 was thought to play a central role in the outbreaks. This role, however, could not be substantiated. Five pigs of the export collection centre were from farm 92-04. The farms of origin of the other four pigs were serologically negative for SVDV antibodies. Therefore, the only epidemiological link that can be substantiated is from farm 92-04 to 92-05.

Farm 92-06, the second fattening farm, showed more or less the same distribution pattern as fattening farm 92-03 (Figure 3). In most pens with

seropositive pigs, all pigs were seropositive. In 11 of the 88 serological positive pigs, only IgG was found, indicating that these pigs were infected at least 49 days (based on D_{50}) before sampling. At the time of the outbreak, it was believed that the firm who had transported piglets from farm 92-01 and 92-02 was most likely responsible for the infection of 92-06. The same firm also transported piglets to farm 92-06, 109 days before SVD was diagnosed. The piglets entered the section in which 52 out of 53 pigs were only IgM positive. Based on this result, the contact of 109 days before is very unlikely the cause of the infection.

The last SVD outbreaks in the Netherlands were in 1994. Two export collection centres, and a small fattening farm became infected. All three infections could be traced back to one source. The route of introduction could not be revealed. One pig on the fattening farm had already lost its IgM antibodies, so that farm had been infected for a much longer period than was thought in 1994. Therefore, this fattening farm may have been the cause of the SVD infection on the first collection centre (94-01).

For the initial diagnosis of the first cases of SVD in 1992, we had to rely on the farmer and his practitioner. In the three breeding farms involved, the infection was noticed by the farmer and/or his veterinarian. In all three cases, lesions on the udder or vesicles on the feet of piglets were observed. In none of the four fattening farms, lesions were observed by the farmer. This demonstrates that in older pigs in an environment where the feet are mostly covered with dung, an SVD infection is likely to be missed. Small piglets and sows on the other hand, are often handled by the farmer and lesions are more likely to be spotted. Because an SVD infection can go unnoticed, it can be argued that a serological monitoring system is necessary. An introduction of SVDV, however, will probably not be limited to only fattening farms where the infection is likely to be missed. Therefore, the design of a valid serological monitoring system for SVD is only possible when knowledge about the structure of contacts between pig farms is available. A comparison between the chances of finding an infection by the monitoring system or by normal clinical inspection can then be made.

In summary, five newly developed isotype specific ELISAs were validated, the IgM and IgG ELISA were very useful to elucidate the epidemiology of SVD, but accurate tracing of contacts is essential for an effective combat of infectious diseases. Record keeping of contacts by the farmer, pig traders, lorry drivers and veterinarians is essential, as well as good identification and registration of farm animals.

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Chapter 7

Swine vesicular disease singleton reactors: results of SVD antibody surveillance in the Netherlands 1993 - 1999

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Abstract

Sera collected in the nation-wide serosurveillance programme for SVD antibodies in the Netherlands are screened at the Regional Animal Health Service by a monoclonal based SVD ELISA and sent to the National Reference Laboratory for confirmation by the VNT. Although the percentage of false positive (ELISA and VNT) reactions is between 0.04 and 0.05, over 300 of these so-called "singleton reactors" were encountered in 1998 and 1999 due to the large numbers of pigs (over 600,000) tested each year. To study the cause of these singleton reactors two sets of sera were selected. The first set, positive in the screening ELISA, was tested for IgM and IgA specific antibodies. The second set of sera had high titres in the SVDV neutralisation test and was tested for neutralising antibodies against Coxsackie B5 virus. Sera from nine pigs experimentally infected with one of both SVDV strains or vaccinated with one of the two Coxsackie strains served as controls in these neutralisation tests. None of the screening ELISA positive sera contained IgA antibodies and 6 out of 8 neutralisation test positive singleton reactor sera contained IgM antibodies. In the SVDV neutralisation positive sera the highest titres were found against SVD isolate UKG/27/72. The neutralisation titres against both Coxsackie B5 strains varied widely. There was, however, no indication that these singleton reactors were caused by Coxsackie B5 infections. Based on these findings it is unlikely that a simple laboratory test to identify a singleton reactor can be developed. The number of singleton reactors can, however, be decreased by increasing the cut-off in the virus neutralisation test, without seriously affecting the sensitivity of the surveillance system.

1 Introduction

Swine vesicular disease (SVD) is a contagious disease caused by an enterovirus (Nardelli et al. 1966). The symptoms of SVD are indistinguishable from those observed after a foot-and-mouth disease virus infection, and therefore it is listed by the Office International des Epizooties (OIE) as a list A disease. The Netherlands was confronted with 6 outbreaks of SVD in 1992. Because trading partners wanted proof that no SVD infected pigs were exported a compulsory serosurveillance programme was adopted (RBD = Regeling Bedrijfscontrole Dierziekten). Each pig herd is inspected by a veterinarian three times a year and blood samples of 12 randomly selected pigs are collected, and tested for antibodies against SVDV. The sample size was based on a test sensitivity of 90%, to detect a farm of infinite size with a 25% seroprevalence at a 95% confidence level (Martin et al. 1992). The programme started in September 1993, and since February 1994 no infected farms have been detected.

During the serosurveillance programme a considerable amount of false positive reactions were encountered. These false positive reactions are mostly limited to one animal on a farm, and in such case are called "singleton reactors". Because SVD is an OIE list A disease each positive serological result should

arise suspicion of being an outbreak and has to be handled as such. In 1993 the European Community adopted a single reference serum for SVD (RS 01.04.93), which should score consistently positive in every serological test for SVD performed within the European Community (Mackay et al., 1996). Countries could, however, use a different cut-off if the test was used in a national serosurveillance programme. Based on this exception, Dutch farms with one low-positive serum (SVDV neutralisation titre less than two times the mean titre of RS 01.04.93) in the serosurveillance programme are considered negative. Each time one or more positive or two or more low-positive samples are found, an official veterinarian visits the farm and takes new blood samples from the seropositive pig and its penmates. Additional samples are taken to complete the total number of samples needed to detect an SVD antibody prevalence of 5% with 95% confidence. Until the results of the retest are available the farm is not allowed to sell pigs.

Virus neutralisation tests in general are considered to be very specific, nevertheless false positive reactions can be found. The cause of the SVDV singleton reactors is not known. De Clercq (1998) described a test system using the virus neutralisation test (VNT), a monoclonal antibody based competitive ELISA and an IgM ELISA to reduce the number of singleton reactors. In that study all singleton reactor sera scored positive in the IgM ELISA, but had a low IgM titre. This supported the hypothesis that low-titre, cross-reactive IgM antibodies were the cause of the singleton reactor phenomenon. De Clercq (1998) considered all VNT and ELISA positive sera that were negative for IgG antibodies and had a low IgM titre to be singleton reactors. Low IgM titres in absence of IgG antibodies can, however, also be found shortly after an SVD infection. Furthermore, the agent supposedly causing these cross-reactions was not identified.

Coxsackie B5 (CB5) and SVD are genetically and antigenically very closely related (Graves, 1973; Brown et al., 1973; Knowles et al., 1997), and CB5 virus can infect pigs (Lai et al., 1980). Natural CB5 virus infections of pigs, however, have never been reported, but an incidental infection of pigs by contact with humans can not be excluded.

We selected two sets of singleton reactor sera and tested one set for SVDV specific IgA and IgM antibodies, and the other for neutralising antibodies against two strains of SVDV and two strains of CB5 virus, to see whether a consistent reaction pattern within these singleton reactors could be found which could help to identify a common cause for these single reactors.

2 Materials and methods

2.1 Cells and viruses

IBRS-2 (de Castro, 1964) cells were grown in Eagles medium supplemented with 2% protein hydrolysate and 5% foetal bovine serum (FBS). PK-15 cells (American Type Culture Collection CCL33) were grown in Earle minimal essential medium supplemented with 5% FBS. The 1972 SVD virus (SVDV) isolate from the UK, UKG/27/72, which is the standard virus strain for

SVD serology in Europe (Mackay et al. 1996), was obtained from the European reference laboratory at Pirbright. SVDV isolate NET/1/92 was isolated in our laboratory from specimens from pigs suspected of vesicular disease from the first outbreak in 1992. The two CB5 virus isolates, Dekking (isolated in 1957) and 94-2663 (isolated in 1994) were kindly provided by dr. T.G. Kimman (R.I.V.M. Bilthoven, the Netherlands).

2.2 Sera

Since February 1997 all sera collected within the framework of the SVD serosurveillance programme are tested at the Regional Animal Health Service in Boxtel using the SVDV antibody ELISA described by Chenard et al. (1998). Before February 1997 the direct liquid phase blocking ELISA (Dekker et al., 1995) was used. ELISA positive samples are sent to our laboratory for confirmation in the VNT.

Forty-seven sera positive in the SVDV antibody ELISA were selected randomly from the sera sent for confirmation in the VNT. These sera were tested in an SVD specific IgA and IgM ELISA (Dekker et al., 1999). Forty-eight singleton reactor sera with high VNT titres were selected for testing in the VNT against SVDV isolates UKG/27/72 and NET/1/92, and CB5 isolates Dekking and 94-2663

Three week postinfection sera from 9 experimentally infected pigs using SVDV isolate UKG/27/72 , NET/1/92, or CB5 isolate Dekking, and three week postvaccination sera from 4 pigs vaccinated with an experimental CB5 vaccine isolate Dekking or 94-2663, were included in the VNT against the two SVD and CB5 isolates, as positive controls on the cross-reactions between SVDV and CB5 virus.

2.3 ELISAs

The direct liquid phase blocking ELISA, the SVDV antibody ELISA and the SVDV specific isotype ELISAs have been described elsewhere (Dekker et al., 1995; Chenard et al., 1998; Dekker et al., 1999)

2.4 Virus neutralisation test (VNT)

Starting with a 1:50 dilution, 50 µl of serial two-fold dilutions of each serum were mixed with 50 µl of SVD virus suspension containing 30-300 TCID50 of SVDV isolate UKG/27/72 and incubated for 1 h.
All dilutions were made in Earle minimal essential medium supplemented with 5% FBS and antibiotics. SVDV isolate UKG/27/72 adapted to the PK-15 cell line was used for this purpose. After PK-15 cells were added, the plates were incubated for 4 days. Next the plates were emptied and rinsed with tap water, the monolayers were fixed and stained with a solution of amido-black and acetic acid and the test was read macroscopically for cytopathic effect. All incubations were made at 37°C in an humidified atmosphere conta ining 5% CO_2.

Table 1: The Dutch SVD serosurveillance programme between 1994 and 1999: Number of samples collected and found positive in the virus neutralisation test.

Year	Screening					Tracing				
	Number of	Low positive*		Positive		Number of	Low positive		Positive	
	Samples	number	%	number	%	samples	number	%	number	%
1994	804890	2295	0.29	343	0.04	34374	421	1.22	69	0.20
1995	760518	1935	0,25	570	0,07	37115	963	2,59	143	0,39
1996	719271	1453	0,20	349	0,05	21416	316	1,48	133	0,62
1997	602023	315	0,05	214	0,04	12921	80	0,62	42	0,33
1998	683407	229	0,03	151	0,02	9132	61	0,67	36	0,39
1999**	334445	82	0.02	68	0.02	4358	29	0.67	9	0.21

* VNT titre < 2.3 ^{10}log

**Data till 31 July 1999

The titres were expressed as the reciprocal value of the serum dilution that prevented virus growth in 50% of the duplicate wells. In each test the virus suspension and a positive control serum were titrated. Up to September 1996 sera with a titre of 2.0 - 2.3 (^{10}log) were considered low-positive, thereafter the threshold was raised to 2.15. Sera with a titre above 2.3 were considered positive. The mean titre of the European reference serum (RS 01.04.93) was 2.19 ± 0.22 ^{10}log (n = 51).

The neutralisation tests on the 48 high-positive singleton reactor were performed in a similar manner. In these tests, however, IBRS-2 cells instead of PK-15 cells were used, because SVD stain NET/1/92 did not grow on PK-15 cells.

3 Results

During the 6 years of the serosurveillance programme for SVDV antibodies 600,000 - 800,000 sera were examined each year, in total over 4 million sera. Since the outbreak of classical swine fever in 1997 the number of pig farms has decreased in the Netherlands and consequently the number of sera tested (Table 1).

Table 2: Virus neutralisation titres obtained with 3-week postinfection and post-vaccination sera

Group	Virus strain used	Virus used in the VNT			
		SVD		CB5	
		UKG/27/72	NET/1/92	Dekking	94-2663
SVD post-infection	UKG/27/72	3.9	3.3	2.85	2.4
	UKG/27/72	3.9	3.3	3.15	2.7
	UKG/27/72	≥4.05	3.45	2.85	2.85
	NET/1/92	3.3	3.9	2.7	2.25
	NET/1/92	3.3	3.6	1.35	1.8
	NET/1/92	3.15	≥4.05	1.8	2.1
CB5 post-infection	Dekking	0.6	N.T.*	1.5	0.9
	Dekking	1.2	0.9	1.2	<0.6
	Dekking	0.75	N.T.	1.05	<0.6
CB5 post-vaccination	Dekking	≥4.05	3.45	≥4.05	3.75
	Dekking	3.3	2.85	3.75	3.0
	94-2663	1.65	0.9	1.95	1.95
	94-2663	2.7	1.8	3.0	3.3

*N.T. = not tested

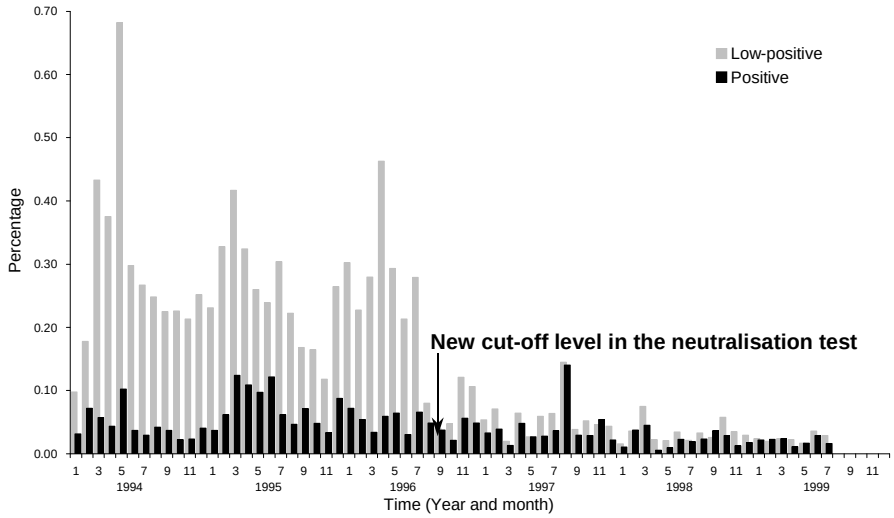

Figure 1: Percentage of positive and low-positive VNT titres found in the serological serosurveillance programme for SVDV in the Netherlands. After September 1996 the cut-off level for low-positive sera was used in the VNT was raised by 0.15 ^{10}log to 2.15.

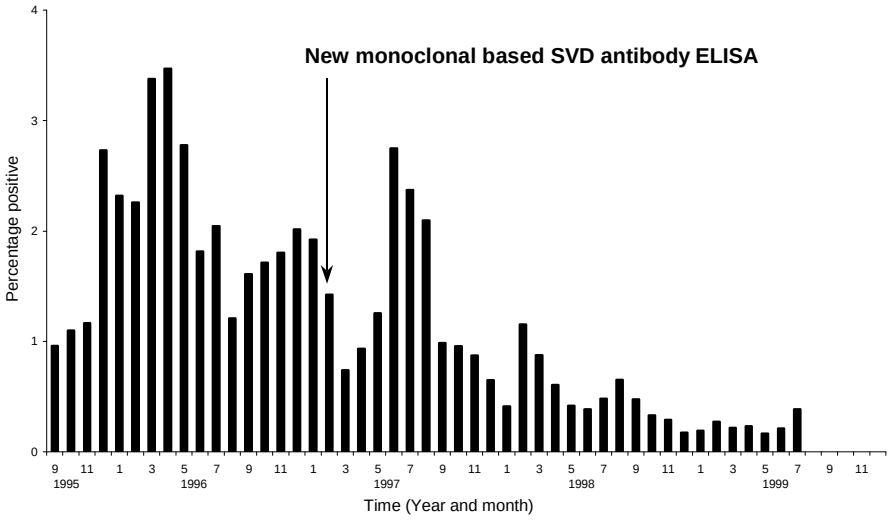

Figure 2: Percentage of ELISA positive samples in the serosurveillance programme for SVDV antibodies in the Netherlands. After February 1996 a new monoclonal based SVDV antibody ELISA (Chenard et al, 1998) was used.

After the increase in the cut-off used in the VNT (September 1996) the percentage of low-positive reactions dropped (Figure 1). On average 0.269% low-positive sera were found before September 1996, and 0.044% thereafter. Changing from the direct liquid phase blocking ELISA to the new monoclonal based SVDV antibody ELISA resulted in a lower percentage of positive reactions in the ELISA (Figure 2). The average percentage of sera positive in the ELISA dropped from 1.97% to 0.74%. Sera with positive reactions in the VNT changed from 0.055% before 1 February 1997 to 0.027% after that date. In sera collected as a consequence of tracing serologically positive animals, the average percentage of positive sera changed from 0.37% to 0.32% over the same period.

Forty-seven sera from the SVD serosurveillance programme, positive in the monoclonal based SVDV antibody ELISA, were tested in the SVDV specific IgA and IgM ELISA. All sera were negative in the IgA ELISA, and 14 were positive in the IgM ELISA. Of the 47 sera, eight were positive in the neutralisation test and three were low positive. Six of eight VNT positive sera were positive in the IgM ELISA and all three sera with low VNT titres had IgM antibodies. Out of the 36 VNT negative sera, five were positive in the IgM ELISA.

The VNT titres on the postinfection and postvaccination sera with different viruses are shown in Table 2. In all cases the homologous titre was the highest, but in one pig infected with CB5 virus the heterologous titre against SVD strain UKG/27/72 was equal to the homologous titre.

The 48 VNT positive singleton reactor sera shown in Figure 3 had all the highest titre against SVDV isolate UKG/27/72. The VNT titre against SVDV isolate NET/1/92 was, in approximately 80% of the cases, higher than the titres obtained with one of the CB5 strains (Figure 3). The titres against the two CB5 virus isolates were in most case equal, only in 15% of the sera the titre against CB5 isolate Dekking was significantly ($\geq$ 4 fold) higher than that against CB5 isolate 94-2663. In approximately 10% of the sera the titre against isolate 94-2663 was significantly higher compared to the titre against isolate Dekking. The titre obtained with one of the CB5 isolates was sometimes only slightly lower than that obtained with SVDV isolate UKG/27/72, but could also be absent (Figure 3).

The serosurveillance programme resulted in the detection of one outbreak (94-02). At that time, February 1994, two sets of samples from the same farm arrived in the laboratory on the same day. The first set of samples were taken within the framework of the SVD serosurveillance programme and the second set taken as a consequence of tracing the contacts of an SVDV infected export collection centre. In both sets a high number of sera, 7 out of 8 and 17 out of 21 respectively, were found positive.

On a few occasions a farm was found with a high number of positive reactions. On one of these farms a non-SVD enterovirus was isolated. Experimental infection of pigs with this isolate, however, did not produce SVD cross-reacting antibodies.

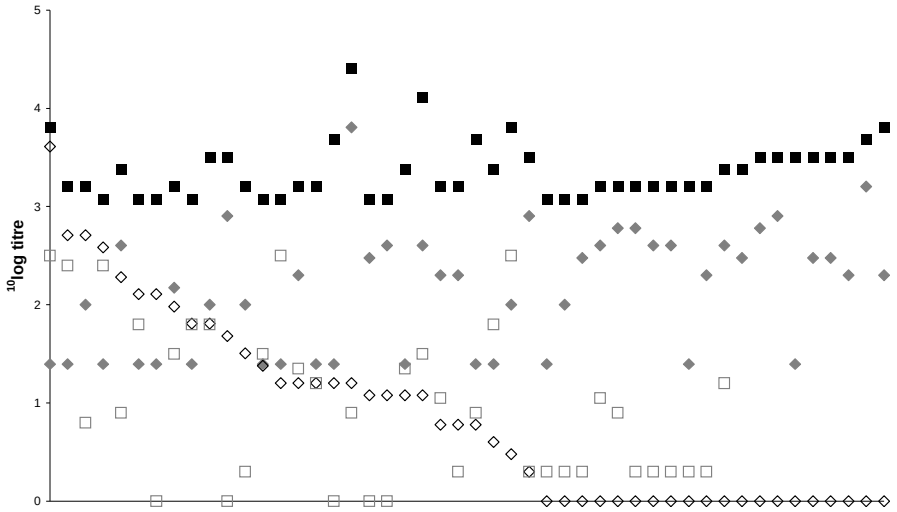

Figure 3: Virus neutralisation titres of 48 singleton reactor sera, tested against SVDV isolates UKG/27/72 (■), NET/1/92 (◆), and Coxsackie B5 virus isolates Dekking (□), and 94-2663 (◇). The sera are ranked by the titre obtained with Coxsackie B5 virus isolate 94-2663

4 Discussion

The initial sample size in the serosurveillance programme was based on a test with a sensitivity of 90%. By changing the ELISA from an indirect type to a direct type liquid phase blocking ELISA and later to the monoclonal based SVDV antibody ELISA the sensitivity is now over 97.5% (Dekker et al., 1995; Chenard et al., 1998). Consequently the sensitivity of the serosurveillance programme has increased. In the EU a serum with a neutralisation titre equal or above the mean titre of the EU reference serum has to be considered positive. This reference serum had a higher neutralisation titre than was used in our test before September 1996. Therefore, we raised the cut-off value of the VNT to this EU standard, reducing the number of false positive reactions significantly. This shows that the VNT titre of the EU reference serum, a dilution of a positive serum, is almost within the range of VNT titres that can be found in non-infected animals. Over 99.9% of the non-infected pigs have SVDV neutralising antibody titres below the EU criterion (Table 1). Therefore, the criterion is valid for tests on individual pigs, e.g. in import- and export screening. Serosurveillance, however, is directed toward the identification of infected herds, not individual infected animals. The goal, detection of herds with a certain antibody prevalence, allows a decrease of sensitivity and consequently increase in

specificity. The decrease in test sensitivity can be compensated by a small increase in the number of animals tested per farm (Figure 4, Martin et al., 1992).

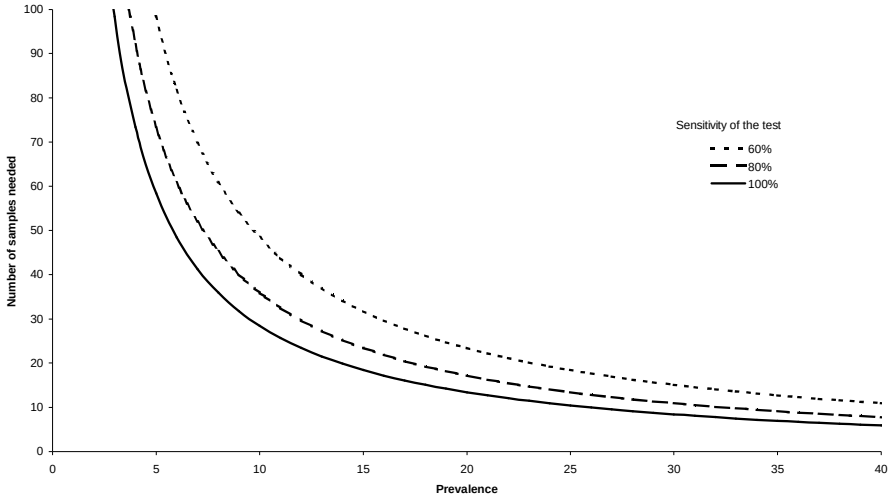

Figure 4: Relation between sensitivity of the test, prevalence and numbers of samples to detect a disease with 95% confidence level. Poor sensitivity can be compensated by a larger number of animals sampled (Martin et al., 1992).

The number of 12 samples in the Dutch serosurveillance programme is based on a sensitivity of 90% (95% confidence level). On average 88% (range 83 - 100%) of the VNT positive sera collected in the 1992 and 1994 outbreaks had a VNT titre equal to or above 2.45 (^{10}log), 2 times the titre of the European reference serum. If the cut off in the VNT had been set to 2.45 instead of 2.15 (^{10}log) and 12 samples had been taken on those farms the chance of detecting the outbreak would have decreased with 0.2% (range 0 - 1.2%), from 91.7 to 91.5%. Cut-offs for diagnostic tests used in serosurveillance should therefore not be based on the detection of a single weak positive serum, but on analysis of the sensitivity and specificity determined on a *panel of post-infection sera*, preferably collected from outbreaks.

The decrease in positive reactions in the ELISA, from 1.97% to 0.74%, after changing to the monoclonal based SVDV antibody ELISA, had also consequences for the percentage of positive reactions found in the VNT, which changed from 0.055% to 0.027%. The sera collected as a consequence of tracing serologically positive animals were directly tested by VNT. Changing to the monoclonal based SVDV antibody ELISA therefore had no influence on the results obtained with these sera. The percentage of positive reactions in these samples remained almost constant, changing from 0.373% to 0.322%. So the

use of the new monoclonal based SVDV antibody ELISA did not only reduce the number of false positive ELISA reactions (sera that did not react positively in the VNT), but also the percentage of VNT positive sera. The sensitivity, however, of both the direct liquid phase blocking ELISA and the monoclonal based SVDV antibody ELISA, is comparable (> 97.5%), so positive farms would not have been missed.

De Clercq (1998) showed that singleton reactor sera could be identified based on their reaction patterns in SVD antibody specific tests. In our set of sera, none of the singleton reactors was positive in the SVDV specific IgA ELISA. However, almost half of the sera from infected pigs collected on outbreak farms were also negative (Dekker et al., 1999). Therefore, absence of SVDV specific IgA antibodies can not be used to identify a singleton reactor. De Clercq (1998) showed that singleton reactors could be identified by a low IgM titre. In our limited set, however, only 4 of the 6 singleton reactor sera with high VNT titres were positive in the IgM ELISA. This may be explained by the fact that we used a different IgM ELISA, or a different set of sera. Further investigation into the possible use of the IgM, IgA and IgG ELISAs is necessary.

We also investigated whether false positive reactions were due to an infection with CB5 virus. The postinfection and postvaccination sera, shown in Table 2, had in all cases the highest titre against the homologous virus. In all pigs infected with SVDV the titre against the heterologous SVDV isolate was higher than the titre against any of the CB5 isolates. But in the pigs infected or vaccinated with CB5 isolate Dekking, the heterologous SVDV titre against UKG/27/72 was often higher than the titre against the heterologous CB5 isolate. The old CB5 isolate from 1957, therefore, seems to be more related to the old SVDV isolate UKG/27/72 than to the 1994 CB5 isolate 94-2663. This confirms the observation of Brown et al. (1976), who found that the two CB5 isolates he tested were more closely related to SVDV than to each other. In the set of 48 singleton reactors (Figure 3) we found high neutralising titres to SVD isolate UKG/27/72. This was expected, because this isolate was used in the standard neutralisation test, and sera with high titres were selected. No serum was found with a higher titre to CB5 virus than to SVDV, which was the case in the CB5 infected and vaccinated pigs (Table 2). So there was no indication that CB5 virus infections had taken place in the pig population. Interestingly the titre difference obtained with UKG/27/72 and the CB5 isolates varied in the set of VNT positive singleton reactor sera (Figure 3). Some sera had antibodies against SVDV and CB5, while others had antibodies only directed against SVDV. This finding makes it unlikely that a single cross-reacting agent causes the singleton reactor phenomenon. The finding of a non-SVDV enterovirus on one of the farms with approximately 10% low positive singleton reactor sera gave rise to the hypothesis that this virus was the cause of these singleton reactors. Experimental infection with this non-SVD enterovirus isolate did not give rise to SVD cross-reactive antibodies. Further research into the causes of the singleton reactor phenomenon is necessary, but it is unlikely that a single cause can be identified.

The serosurveillance programme was capable of detecting the last outbreak (94-02) in the Netherlands. It can be argued, however, that this outbreak was at the same time also detected by the tracing of outbreak 94-01. Outbreaks 94-01 and 94-03 were on export collection centres, where no serum samples are collected, and thus were missed in the serosurveillance programme. Based on the information currently available it is not possible to calculate how quickly an outbreak is detected by the surveillance programme or by clinical suspicion. For this, studies on the transmission of SVD virus between animals, pens and farms are necessary.

After six cases of SVD in 1992 and three in 1994 the serosurveillance programme for SVDV in the Netherlands has proven that the country has been free of SVD since. The current EU reference serum, however, should be replaced by a panel of positive reference sera preferably collected from an outbreak, which can be used to determine the sensitivity of different tests at different cut-off levels.

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Summarising discussion

Pathogenesis, diagnosis and epizootiology of swine vesicular disease

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1 Pathogenesis

In previous experiments, virus could only be isolated from pigs that had been in contact with inoculated pigs for 2 days (Lai et al., 1979). The experiments described in Chapter 1 show that pigs infected by exposure to a contaminated environment developed clinical signs and antibodies as quickly as pigs that were directly inoculated. Virus could be isolated from pigs infected by exposure to a contaminated environment at 1 day post exposure, comparable to results obtained with directly inoculated pigs (Burrows et al. 1974). In the previous experiments the pigs were already housed together at the time of infection. The reason that in those experiments it took 2 days before contact exposed animals became viremic was probably due to extra time needed for the virus to replicate in the inoculated animal before the contact animal was exposed. In our experiments the environment was already contaminated at the time the pigs entered the stable. The speed of infection was probably enhanced by the fact that virus gained entry by skin lesions caused by fighting, because at the time of exposure the social order in the group had to be established. It would therefore be interesting to measure the contribution of fighting in the infection process. For that purpose, an experiment could be designed in which two groups of pigs are infected by contact to a contaminated environment. In the first group the social order has been established before contact exposure and in the second group pigs originating from different pens of the same farm will be mixed during contact exposure.

From the results described in Chapter 1, it is clear that already 1 day after contact to an SVDV contaminated environment, spleen and kidney contained infectious virus. The pigs on the Italian abattoir (see introduction) could therefore also have contracted the disease on the premises of the slaughterhouse. Together with the fact that the serosurveillance programme proved that the Netherlands had been free of SVD between 1992 and 1994, it is almost impossible that pigs from the Netherlands were the cause of SVD infections on Italian abattoirs.

Because high titres of SVDV were found in several lymphoid tissues (Chu et al., 1979; Lai et al., 1979; Chapter 1) we were interested whether the virus was able to replicate in those tissues. Initial experiments with tissues infected *in vitro* with SVDV produced inconclusive results (data not shown). Therefore, we developed two new methods, immunohistochemistry (IHC) and in-situ hybridisation (ISH), enabling *in vivo* SVDV replication studies (Chapter 2). Using cytospins of infected cells, more cells were found infected using IHC than ISH indicating a higher sensitivity of the IHC. Using IHC we were able to detect SVDV in the dermis of an infected pig 4.5 h after infection. In the experiment the epidermis was damaged before infection, so this might have been the cause of the infection of dermal cells. However, it is not known whether damage of the epidermis is essential for infection. The pigs in Chapter 1 all had epidermal lesions due to fighting. Lai et al. (1979) found SVDV specific fluorescence in cells of hair follicles of infected pigs 2 days after contact exposure. This indicates that virus trapped in hair follicles might gain entry into the animal. Therefore,

experimental infection of an intact epidermis could be compared to infection of a lacerated epidermis, where especially the role of the cells in hair follicles could be studied.

2 Diagnosis

Serosurveillance is of great value to discover undisclosed foci of infection and to prove absence of disease. Therefore a specific, sensitive and easy to perform serological test was needed. In Chapter 3 an improvement in specificity of a previously described ELISA based on polyclonal antibodies is reported. With the use of monoclonal antibodies (Chapter 4) a more specific ELISA was developed (Chapter 5), which is therefore currently used in the SVD serosurveillance programme. Among the newly produced monoclonal antibodies some did not neutralise the virus, therefore a new method for epitope mapping on picornaviruses was developed (Chapter 4). Using this method also epitopes of non-neutralising monoclonal antibodies can be mapped. For seven new monoclonal antibodies we mapped the region where the epitope was located. These regions consisted of approximately 80 amino acids. Further refinement is necessary to pinpoint more precisely the epitopes recognised by these monoclonal antibodies.

3 Epizootiology

The SVD serosurveillance programme in the Netherlands provides ample proof that no SVD outbreaks have been missed since the last outbreak in 1994 (Chapter 7). According to the animal health code of the Office International des Epizooties (Anonymous, 1999), a country may be considered free from SVD when it has been shown that SVD has not been present for at least the past 2 years. This period may be 9 months for countries in which a stamping-out policy is practised. Based on this definition, which is similar for most List A diseases, continuous serological surveillance for SVD or any other List A disease is not necessary. The cost of the serosurveillance programme for SVD is high, over 3 million Euro per year. Therefore it is essential to know the effectiveness of the programme. In most List A diseases clinical signs will be evident in case of infection. Serosurveillance will, in those cases, not shorten the time between introduction of the agent and detection, and is therefore not useful as an early warning system. In the case of SVD, clinical disease may go unnoticed, in such a situation serosurveillance might be effective. Serosurveillance led to discovery of one SVD outbreak in 1994 (94-02), simultaneously with tracing, whereas tracing back an outbreak in Italy led to the discovery of all three outbreaks. Based on the isotype specific responses, pigs in outbreak 94-02 had been infected for at least one month (Chapter 6). So, based on these observations, the serosurveillance programme had not contributed to early detection. The current serosurveillance programme does not guarantee that every pig that is exported is free of SVD, because the farm could have become infected after the sampling, or have been missed because the seroprevalence was too low.

Therefore the pre-eminent question remains; when SVDV is introduced into the Netherlands what is the chance that the infection is transmitted from one farm to another before the disease is diagnosed by the serosurveillance programme or by clinical suspicion? This is the kind of risk assessment referred to by the World Trade Organisation in article 5 paragraph 1 of the agreement on the application of sanitary and phytosanitary measures (Anonymous 1994). Risk assessment should not only be performed for SVD, but for most other diseases. However to be able to do this, more information must be available on normal contacts between farms and the transmission rates of the disease. For this purpose not only the transmission rate within farms, but also the transmission rate between farms should be studied. In Chapter 6, we tried to obtain some of this information by analysing the 1992 and 1994 SVD outbreaks with IgM and IgG specific ELISAs. Although the results are very clear with regard to the transmission within the farm, SVD is a pen disease; almost no extra information on the duration of the infection before discovery was obtained. IgM and IgG ELISAs can help tracing contacts, but they can not replace it. In disease control, tracing of contacts is very essential to identify other outbreaks, but is often hampered by the absence of reliable data. Especially in diseases like SVD or classical swine fever where the time between introduction and discovery can be very long, contacts that may have caused the introduction of the virus are not always remembered. A system of compulsory logging all animal contacts on a farm will help tracing those contacts. Such a system would not only be beneficial in case of an SVD outbreak, but also in case of outbreaks of other diseases.

Research into the causes of the so called "singleton reactor phenomena" was not successful (Chapter 7). No evidence was found that CB5 virus was the cause of this phenomenon. Low IgM titres, however, suggested that something else induces antibodies, which cross-react with SVD. Our results indicated that there is probably not one cause for this phenomenon.

Testing over 600,000 pigs per year resulted in 380 to 2600 positive VNT reactions. Based on EU regulations all farms with SVD serologically positive pigs should be considered suspected for SVD and should be treated likewise. In 55 - 87% of the positive results only low virus neutralisation test (VNT) titres were found. Only farms with high positive results or farms with two or more low positive sera were considered suspected and were retested. Analysis of the neutralisation titres of sera collected at the different SVD outbreaks in the Netherlands showed that on average 88% (83 - 100%) of the positive sera had a VNT titre two times higher than the titre of the European reference serum (RS 01.04.93). Using this higher cut-off (more than two times the titre of the European reference serum), farms with a seroprevalence of 25.7% can be detected in the Dutch serosurveillance programme at a 95% confidence level. If the cut-off had been set at the level of the European reference serum farms with a seroprevalence of 22.7% could have been detected (95% confidence level) (Martin et al., 1992). The chance of detecting the 1992 and 1994 outbreaks was only 0.2% (range 0 - 1.2%) lower (Chapter 7). Using the higher cut-off in the VNT we reduced the number of false positive reactions with more than 50%, without seriously affecting the herd sensitivity. Therefore, the cut-off of a

serological test should not be based on its capacity to detect a single low-positive serum, but be based on the evaluation of the sensitivity and specificity on a panel of international standard sera. Such an international set of standard sera should reflect the situation that can be found in an outbreak. Using this panel every laboratory should validate the sensitivity of their test in order to prevent international disputes.

Concluding remarks

One of the major reasons for the distrust between European trading partners was the absence of a European standard for SVDV serology. Due to differences between titres obtained in different laboratories, different cut-off levels were used. Therefore the sensitivity of the tests with a high cut-off was questioned. Without a European standard for serology and proficiency testing of all European laboratories this problem could not be solved. Current World Trade Organisation regulation (Anonymous, 1994) allow governments to implement zoosanitary measures, irrespective of the fact that a disease is placed on the List A or B of the Office International des Epizooties. To fulfil article 2 of the WTO agreement on the application of sanitary and phytosanitary measures that zoosanitary measurers do not unjustifiably or arbitrarily discriminate between members, good standards for disease surveillance and diagnosis are needed for all diseases. SVD might also be present in former East-European countries, therefore laboratories in future member states should be included in the EU proficiency tests, not only for SVD, but for all important diseases of livestock.

Because serosurveillance for diseases with overt clinical signs is superfluous, investigation of cases of animal disease is still the most important instrument of surveillance for such diseases (Anonymous 1999). Therefore reporting unusual cases of animal disease should be encouraged. Farmers and veterinary practitioners should be informed, on a regular basis, about clinical signs, notification and control measures of all notifiable diseases. In case of serious suspicion a swift clinical examination by a (trained) official veterinarian should be carried out. Control measures should only be implemented when there is suspicion of a serious disease.

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Dutch Summary

Pathogenesis, diagnosis and epizootiology of swine vesicular disease

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Vesiculaire varkensziekte of swine vesicular disease (blaasjesziekte) (SVD) is een besmettelijke virale aandoening bij varkens, die veroorzaakt wordt door swine vesicular disease virus. De virusinfectie veroorzaakt blaren op de huid en slijmvliezen van varkens. De blaren worden meestal waargenomen op de overgang van de huid naar de klauwen, maar kunnen zich ook op andere delen van de poten of aan de buikhuid voordoen. Blaren op de neus en in de mond kunnen ook worden waargenomen, maar komen minder vaak voor. Echter blaren op de neus worden vaak eerder gezien door de veehouder dan blaren op de poten, omdat de varkens niet altijd kreupel zijn tengevolge van de blaren op de poten. Omdat de verschijnselen van SVD bij een individueel varken niet zijn te onderscheiden van die van MKZ is SVD ook aangifteplichtig gemaakt, niet alleen in Nederland, maar ook in de EU en vele landen daarbuiten. In 1992 werd er in Nederland na 17 jaar afwezigheid voor de tweede maal een uitbraak van SVD gediagnosticeerd. Deze uitbraak had belangrijke economische consequenties, niet vanwege directe verliezen door groeivermindering of sterfte, maar omdat de export van varkens, varkensvlees en varkensvlees producten geblokkeerd werd. De export werd op last van de EU in 1992 stilgelegd; dit kostte destijds ca. 35 miljoen gulden. Normaliter wordt de export slechts stilgelegd in het gebied waar de uitbraak zich voordoet. In 1992 en 1993 werden er in Italië Nederlandse varkens aangetroffen met SVD. Dit leidde tot de veronderstelling dat Nederland verantwoordelijk was voor deze uitbraken in Italië en daarom heeft destijds de EU besloten export van varkens en varkensvlees uit geheel Nederland tijdelijk niet toe te staan, totdat er garantie kon worden gegeven met betrekking tot het vrij zijn van de ziekte.

De uitbraak en de daaraan gelieerde problemen waren aanleiding tot het stellen van een aantal vragen: Kon de besmetting van Nederlandse varkens in Italië ook het gevolg zijn van een primaire infectie in Italië? Hoe zou kunnen worden aangetoond dat Nederlandse varkens niet de oorspronkelijke bron waren van deze infecties? Hoe was SVD in 1992 in Nederland geïntroduceerd en hoe verliep de verspreiding?

Het onderzoek beschreven in dit proefschrift probeert onder andere op deze vragen een antwoord te vinden. Dit is gedaan door:

1) De bestudering van de ontwikkeling van het ziektebeeld in een situatie die het transport en aankomst in Italië nabootst (Hoofdstuk 1)
2) De mogelijkheden van serologische diagnostiek te verbeteren (Hoofdstuk 3 en 5)
3) Door epizoötiologisch onderzoek (Hoofdstuk 6 en 7).

Op basis van de resultaten beschreven in Hoofdstuk 1, werd aangetoond dat het virus zich heel snel kan verspreiden binnen een stal. Hoe de verspreiding in het lichaam plaatsvindt is echter nog onbekend. Voor dit doel is een aantal technieken gevalideerd om dit te bestuderen (Hoofdstuk 2). De epitopen op het virus die door monoclonale antilichamen herkend worden zijn voor het inzicht in de structuur van het virus van belang. Een nieuwe methode om deze epitopen te bestuderen staat beschreven in Hoofdstuk 3.

Zoals boven genoemd was het aantonen van SVD virus in Nederlandse varkens in Italië één van de redenen dat de export van Nederlandse varkens in 1992 en 1993 door de EU werd verboden. Op dat moment leek het aannemelijk dat de oorsprong van het virus in Nederland lag. Echter nagenoeg alle varkens

waarin virus werd aangetoond hadden geen antilichamen tegen het virus, waardoor het idee ontstond dat het virus zich sneller zou kunnen verspreiden in een varken dan in eerdere experimenten was aangetoond. Daarom werden twee experimenten uitgevoerd waarbij de situatie zoals die zich mogelijk in Italië had voorgedaan werd nagebootst (Hoofdstuk 1). Uit deze experimenten werd duidelijk dat het virus zich binnen 1 dag na contact met een gecontamineerde omgeving kan verspreiden in het varken, waardoor de aanname dat Nederlands varkens het virus hadden geïntroduceerd in Italië niet langer houdbaar was.

De experimenten beschreven in Hoofdstuk 1 tonen aan dat het virus in staat is zich binnen 1 dag zich door het varken te verspreiden. Dit leidde tot de vraag: via welke route gebeurt dit. Om dit te kunnen bestuderen waren er nieuwe technieken nodig; de ontwikkeling van deze technieken staat beschreven in Hoofdstuk 2. Er is een aanwijzing dat mogelijk dendritische cellen in de huid een rol spelen bij de verspreiding, maar dit moet nog verder onderzocht worden.

De screening van contactbedrijven tijdens de 1992 uitbraken gebeurde in eerste instantie door middel van klinische inspectie. Volgens de op dat moment beschikbare gegevens zouden SVD geïnfecteerde varkens vaak kreupel zijn en zouden blaren bij inspectie goed zijn waar te nemen. Echter varkens op een bedrijf bevinden zich in een vuilere omgeving met minder licht dan varkens tijdens een experimentele infectie. Er werd door de practici sterk getwijfeld aan de effectiviteit van deze inspecties. Het was aan het buitenland moeilijk hard te maken dat deze inspecties effectief waren. Daarom is besloten de bedrijven in de buurt van de bekende uitbraken serologisch te onderzoeken. De toen beschikbare serologische technieken waren niet geschikt voor het uitvoeren van grootschalige screening, daarom is een ELISA voor grootschalige screening ontwikkeld (Hoofdstuk 3).

De test ontwikkeld in Hoofdstuk 3 voldeed aan de verwachtingen, echter toen door het opzetten van een landelijk screeningsprogramma het aantal monsters toenam ontstond de behoefte aan een specifiekere test. Voor dit doel zijn monoclonale antilichamen gericht tegen SVD virus ontwikkeld. In Hoofdstuk 4 wordt beschreven hoe deze monoclonale antilichamen zijn gemaakt en zijn getypeerd. Eén van deze monoclonale antilichamen werd gebruikt voor de ontwikkeling van een nieuwe test (Hoofdstuk 5), waarmee ca. 50% minder vals positieve reacties worden gevonden.

Eén van de grote vragen tijdens de uitbraak van SVD was; waar komt het virus vandaan, en hoe is het in Nederland verspreid. Uitgebreid onderzoek op de uitbraak bedrijven waar gekeken is naar de contacten die deze bedrijven hebben gehad in de voorafgaande periode leverde niets op. Toen kwam de vraag, zijn we ver genoeg teruggegaan bij het onderzoek. In Hoofdstuk 6 wordt getracht een antwoord te geven op deze vraag. Tot ca. 49 dagen na infectie zijn er IgM antilichamen aanwezig in een geïnfecteerd varken. Met behulp van isotype specifieke ELISA's (IgM en IgG) werd getracht het moment van besmetting van de bedrijven te schatten. Zoals hiervoor aangegeven is een schatting van het infectie tijdstip afhankelijk van het isotype profiel, echter na ca. 49 dagen blijft het profiel gelijk en is er geen betrouwbare schatting van het infectie tijdstip mogelijk. Echter op 4 van de 6 geteste bedrijven (92-03, 92-04, 92-06 en 94-02) waren dieren aanwezig die alleen IgG positief waren en dus meer dan 49 dagen

geleden besmet waren. De infectie op deze bedrijven was dus langer aanwezig geweest dan destijds werd gedacht. Om in de toekomst de tracering van besmettelijke contacten te vergemakkelijken zal een systeem van het verplicht bijhouden van alle contacten op een dierhouderijbedrijf moeten worden opgezet.

In het screeningsprogramma werd ondanks de hele hoge specificiteit van de test toch nog een groot aantal (meer dan 300 per jaar) bedrijven gevonden met positieve reacties. In alle gevallen ging het hier om vals positieve reacties. Omdat deze reacties vaak gevonden worden bij slechts 1 varken op een bedrijf worden ze singleton reactors genoemd. De oorzaak van deze singleton reactors is onbekend, in Hoofdstuk 7 wordt getracht dit op te helderen. Het onderzoek toont aan dat er waarschijnlijk niet één oorzaak voor dit probleem kan worden gevonden. Aanpassing van de grenswaarde in de serologische test kan echter het aantal positieve varkens aanzienlijk verlagen, zonder dat dit ernstige consequenties heeft voor de gevoeligheid. Er bestaat een zwak positief Europese standaard serum waarvan de gemiddelde titer als cutt-off moet worden gebruikt in serologische testen uitgevoerd op individuele varkens, echter een standaard panel waarmee de sensitiviteit van een test kan worden bepaald bestaat niet. Voor de validatie van testen welke gebruikt worden om besmette bedrijven op te sporen is een dergelijk panel noodzakelijk. Voor een goede keuze van de steekproefgrootte tijdens een bedrijfsscreening is inzicht in de sensitiviteit van de test noodzakelijk.

Het onderzoek beschreven in dit proefschrift en het daarmee samenhangende serologische screeningsprogramma heeft er toe bijgedragen dat Nederland aantoonbaar vrij is van SVD infecties. Klachten over verspreiding van SVD virus door Nederlandse varkens zijn de afgelopen jaren vele malen met succes weerlegd met de gegevens uit het screeningsprogramma, zonder dat deze klachten aanleiding waren tot een exportverbod.

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Numbers and Facts

- The Netherlands imported 174,655 pigs and 371,470 cattle in 1998 (FAO database)
- Over 4 million Micronic tubes have conveniently been used to store sera tested for antibodies against SVDV.
- In 1998 the Netherlands had 4645 veterinarians of whom 1916 were registered as veterinary practitioner, and 1273 were female.
- Since the 1992 SVD outbreaks over 60,000 serum samples have been tested in the neutralisation test using Greiner flatbottom 96 wells tissue culture plates.
- On 1 May 1998 4,283,084 head of cattle and 13,445,866 head of pigs inhabited the Netherlands.
- The total value of the export of live animals, meat, milk, eggs and their products was in 1998 8.26 x 10^9 Euro, approximately 2.7% of the Gross National Product.
- The Netherlands exported 1,502,570 pigs and 66,214 cattle in 1998 (FAO database)
- The Netherlands tested over 1.5 million serum samples in the Ceditest ELISA for SVDV antibodies using Greiner high binding ELISA plates.
- 2,4 million cattle and 19.3 million pigs were slaughtered in the Netherlands in 1998.
- Approximately 52,000 cattle farms and 19,345 pig farms were registered in the Netherlands in 1998.
- In 1998 177,300 people were employed in the egg and meat industry in the Netherlands, approximately 1.1% of the total population.
- Approximately 25 animal experiments have been performed on SVD since the outbreak in 1992. In these experiments 179 pigs 4 rabbits and 15 mice have been used. Material and results of 13 of these experiments have been used in this thesis.

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