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Construction of a full-length infectious cDNA clone of swine vesicular disease virus strain NET/1/92 and analysis of new antigenic variants derived from it

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The Dutch swine vesicular disease virus (SVDV) isolate NET/1/92 was one of the first isolates belonging to a new SVDV antigenic group. This strain was completely sequenced and was shown to have 93% similarity with the UKG/27/72 isolate. To enable antigenicity, replication, maturation and pathogenicity studies of NET/1/92, an infectious full-length cDNA clone, designated pSVD146, was prepared. The *in vitro* and *in vivo* biological properties of the virus derived from pSVD146 were studied by analysing antigenicity, plaque morphology, growth curves and virulence in pigs. The epitopes of newly prepared monoclonal antibodies were roughly mapped by fusion-PCR. Fine mapping of epitopes at the amino acid level was achieved by introducing single amino acid mutations in pSVD146. Two new amino acids important in epitope formation were located in VP1; one was mapped in the C-terminal end and the second is thought to be located in the H–I loop. Growth curve and plaque sizes *in vitro* were similar between virus derived from pSVD146 and the parent wild-type virus. In virulence studies in pigs, the lesions score, neutralization titres and the seroconversion rates were comparable between virus derived from pSVD146 and the parent strain. Since virus derived from pSVD146 had the same biological properties as the parent strain NET/1/92, the full-length infectious cDNA clone pSVD146 will be very useful in studies of the antigenicity, virulence, pathogenesis, maturation and replication of SVDV.

Introduction

Swine vesicular disease virus (SVDV) is the causative agent of a highly contagious disease in pigs that causes vesicular lesions in the mouth and on the feet. Symptoms are clinically indistinguishable from those caused by foot and mouth disease virus and swine vesicular disease is therefore classified as a list A disease by the Office International des Epizooties (OIE). SVDV belongs to the family *Picornaviridae*, genus *Enterovirus*. The virus is non-enveloped and has a 30 nm capsid of icosahedral symmetry made up of 60 copies of four proteins, VP1 to VP4 (Murphy *et al.*, 1995; Rueckert, 1996), which encase the RNA genome. The genome of SVDV is a positive single-stranded RNA molecule of approximately 7400 nucleotides in length (Inoue *et al.*, 1989; Seechurn *et al.*, 1990) that codes for a single polyprotein. This polyprotein shows similarity to the human coxsackievirus B5 and poliovirus polyproteins (Rueckert, 1996).

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The first outbreak of SVDV occurred in Italy in 1966 (Nardelli *et al.*, 1968). Since then, several more outbreaks of SVDV have occurred in Europe (Brocchi *et al.*, 1997); the most recent ones occurred in Italy in 2000 (OIE, 2000). In 1992 there were six outbreaks of SVDV in the Netherlands and isolated in the first of these outbreaks was SVDV strain NET/1/92. To enable manipulation of the SVDV strain NET/1/92 genome for further investigation, we sequenced and constructed a full-length infectious cDNA copy of the RNA genome. Since the first construction of an infectious cDNA clone for poliovirus (Racaniello & Baltimore, 1981), many cDNA copies from RNA genomes of members of the *Picornaviridae* have been constructed. These cDNA copies have been used for the development of recombinant vaccines and to study virulence and pathogenicity. Full-length cDNA copies were constructed from two Japanese SVDV strains (J1′73 and H/3′76) (Inoue *et al.*, 1990; Kanno *et al.*, 1998). These two SVDV cDNA copies

were used to study virulence differences between the two strains (Kanno *et al*., 1999). Until now, no full-length cDNA copies of the European SVDV isolate were available. Recently isolated SVDV strains, like NET/1/92, differ significantly at the genetic and antigenic level from the Japanese strains, which were isolated in the 1970s (Brocchi *et al*., 1997; Zhang *et al*., 1999). Based on the monoclonal antibody (MAb) reaction pattern, NET/1/92 was classified into group IV together with all other SVDV strains isolated in Europe from 1992 to 1995 (Dekker *et al*., 2000).

Identifying epitopes is essential for studying virus pathogenesis, epidemiology, virus–host interactions, and for the design of effective vaccines. Using MAb-resistant mutants Kanno *et al*. (1995) and Nijhar *et al*. (1999) showed that three major epitopes could be identified in SVDV similar to the situation seen with poliovirus. However, with MAb-resistant mutants, only epitopes of neutralizing MAbs can be mapped. We therefore tried a different approach to identify epitopes of neutralizing and non-neutralizing MAbs. Using the chimeric SVDV constructed using a fusion-PCR technique we were able to roughly map the epitope regions of eight MAbs, neutralizing and non-neutralizing, which discriminate between the two SVDV strains ITL/1/66 and NET/1/92 (Dekker *et al*., 2000). From the information provided by these roughly mapped epitope regions, we predicted specific amino acids involved in epitope formation. The sites of interest were mutated in the infectious cDNA clone of NET/1/92 produced in this study. We constructed four antigenic variants of the SVDV NET/1/92 strain. Amino acids important for two different epitopes, which have not been described before, were found. The other epitopes found in this study have been previously described for either SVDV or poliovirus. We show the usefulness of this infectious cDNA clone for the fine mapping of the epitopes described by Dekker *et al*. (2000).

Furthermore, we show that the virus derived from the infectious cDNA clone mimics the parent strain regarding growth in cell culture and virulence in pigs, demonstrating that this clone is an excellent tool for further studies of the antigenicity, virulence and pathogenesis of SVDV.

Methods

■ **Viruses and cells.** SVDV strain NET/1/92 was isolated in Lelystad and strain ITL/1/66 was obtained from E. Brocchi (Brescia, Italy). The other SVDV strains used for this study were obtained from the European Reference Laboratory for SVDV (Pirbright, UK). All viruses were grown on IBRS-2 cells using Eagle's medium supplemented with 5% foetal bovine serum and antibiotics. When the cells displayed 90% cytopathic effect, the cell cultures were frozen and thawed. Before use, the virus suspension was clarified by centrifugation at 6000 ***g*** for 10 min. SK6T7 cells expressing the T7 polymerase (Van Gennip *et al*., 1999) were used for transfection of DNA.

■ **Sequencing of NET/1/92.** SVDV strain NET/1/92 was isolated from vesicular lesions and subsequently grown on secondary porcine kidney cells (PK2). From this virus stock, RNA was isolated (Sambrook *et al*., 1989). RT–PCR was performed and PCR products were amplified and cloned into a pGEM-T vector (Promega). The cloned products were sequenced using a double-stranded DNA sequencing method and the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin Elmer). The primer designs were based on the published sequence of SVDV (Inoue *et al*., 1989; Seechurn *et al*., 1990). Data collection was carried out with the ABI PRISM 310 Genetic Analyser (Perkin Elmer). The complete genome was sequenced from two or more individually isolated NET/1/92 RNA genomes, in two directions. To obtain the sequence of the 5′ end of NET/1/92, a 5′ RACE was performed as described previously (Meulenberg *et al*., 1998).

■ **Construction of a full-length cDNA copy.** On the isolated RNA of NET/1/92, an RT–PCR reaction was performed with a primer, pr49, complementary to the poly(A) tail. This 3′ primer, pr49, contained a stretch of 40 T nucleotides and additional restriction sites; 5′ AGATCTGCAGAAGCTTCGATCG$(T)_{40}$ 3′. Amplification was performed using the same 3′ primer and a primer, pr45, at the 5′ end. The primer pr45 contained a T7 promoter (underlined) and some additional restriction sites; 5′ CCCCTGCAGATCTAATACGACTCACTATAGTTAAAACAGCTTGTGGGTTGTT 3′. The PCR reaction resulted in a cDNA copy of the complete NET/1/92 genome. This full-length PCR product was digested with *Eco*RI/*Bgl*II or *Eco*RI/*Pst*I as depicted in Fig. 1 and cloned into pOK 12 (Viera & Messing, 1991) in two steps. From this pOK 12 the *Eco*RV/*Fsp*I fragment containing the T7 promoter was then deleted. The infectious full-length cDNA clone obtained was named pSVD146.

■ **Mutagenesis.** Site-directed mutagenesis was performed in the region of interest (see Table 1 for primers) using a fusion-PCR. Individual parts were amplified with the reverse or the forward mutated primer. The two mutated PCR products were hybridized together and amplified with two primers outside the mutation. The mutated fragments were then digested with *Ngo*MIV and *Alf*II and were reintroduced into pSVD146. After sequence analysis the mutated clones were transfected into SK6T7 cells to obtain mutated virus.

■ **Transfection.** Transfection of 100 ng linearized DNA (*Pvu*I-digested) was performed in SK6T7 cells with lipofectin according the manufacturer's protocol (Gibco BRL). To increase virus titres, the supernatant of transfected SK6T7 was passaged onto IBRS-2 cells 48 h after transfection. Monolayers of SK6T7 cells were used to check the virus production with an immunoperoxidase monolayer assay (Wensvoort *et al*., 1986) in which swine anti-SVDV hyper-immune serum (Dekker *et al*., 1995) was used to detect virus.

■ **MAb screening ELISA.** ELISA plates (Costar) were coated overnight at 4 °C with a predetermined dilution of rabbit antibodies directed against SVDV in coating buffer (sodium carbonate buffer, pH 9·6). After washing with tap water containing 0·05% Tween 80, antigen (virus) was added and incubated for 1 h at 37 °C. MAbs conjugated with horseradish peroxidase were added after washing and the plates were then incubated for 1 h at 37 °C. The MAbs used have previously been described by Dekker *et al*. (2000). The plates were then washed and 100 µl of chromogen substrate (TMB/H_2O_2) was added. After 15 min the reaction was stopped by the addition of 100 µl of 1 M H_2SO_4. The plates were read in a spectrophotometer (Titretek) at a wavelength of 450 nm. In each test, a conjugated hyper-immune serum (HIS SVD) was included. This serum was raised against SVDV isolate UKG/27/7 and reacts with all SVDV isolates studied to date.

■ **Growth curves.** Single-step growth curves of virus SVD146 obtained from the pSVD146 construct and SVDV strain NET/1/92 were performed at an m.o.i. of approximately 5 on both IBRS-2 and PK2 cells. The cultures were frozen at different time-points and the virus titres were determined, after thawing, by a standard plaque assay on IBRS-2 cells.

■ **Virulence.** Two groups of five 12-week-old pigs were housed in separate stables in a high containment unit. All pigs were negative for antibodies to SVDV prior to infection. The virus SVD146, obtained from pSVD146, and the parent virus, strain NET/1/92, were plaque-purified on PK2 cells and virus with a titre of 10^5 $TCID_{50}$/ml was used to infect pigs. Approximately 0·1 ml virus was inoculated into five separate injection sites of the bulb of the left outside heel. On days 0, 2, 5, 7, 12, 16 and 21 after infection, the pigs were anaesthetized and the lesions on the feet and in the mouth were scored (Burrows *et al*., 1974). Serum and faecal samples were taken on each day that the pigs were examined for lesions. Sera were examined for neutralizing antibodies as described by Dekker *et al*. (1995).

Results

Sequence of NET/1/92

The sequence of NET/1/92 is the consensus sequence of at least two independently sequenced RNA genomes isolated from the plaque-purified virus. The sequence shares 93% identity with UKG/27/72 (used as the reference strain internationally). The nucleotide sequence of NET/1/92 has been submitted to GenBank (accession no. AF268065).

Construction of the full-length cDNA clone pSVD146 and production of derived virus

Digested PCR products representing the complete NET/1/92 genome were successfully ligated and inserted into the pOK 12 vector according to the scheme depicted in Fig. 1. The 5′ end of this product contains the T7 promoter sequence, which was introduced into the terminal 5′ primer (Table 1). This full-length cDNA construct was named pSVD146. The orientation and composition of the inserts were confirmed by restriction analysis and the complete clone was sequenced in

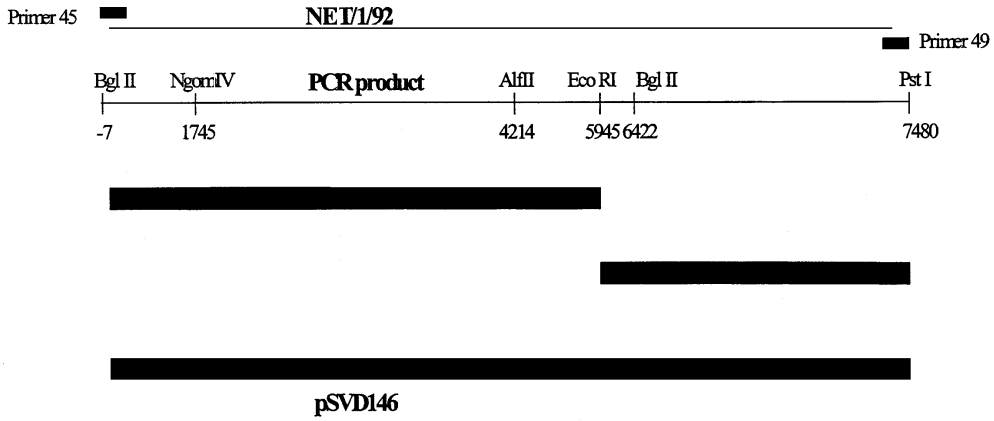

Fig. 1. Construction of the full-length infectious cDNA clone pSVD146. PCR fragments are indicated by the black bars. The restriction sites used are also indicated. The full-length cDNA copy was inserted into the *Bgl*II/*Pst*I site of the pOK 12 plasmid.

Table 1. Sequences of the primers used for mutagenesis and construction of a full-length cDNA copy of the SVDV genome

The mutated nucleotide(s) (underlined) and the codon that the mutation effects are indicated in bold. Amino acid changes are shown in brackets.

Primer	Nucleotide sequence	Nucleotide position
VP1-87 forward	CTGATGGT**GA**CAACTTCGCCT	2699–2719 (G → D)
VP1-87 reverse	AGGCGAAGTT**GT**CACCATCAG	2719–2699 (G → D)
VP1-210 forward	ACCCACT**GGC**GTGACAGAGG	3246–3265 (S → G)
VP1-210 reverse	CCTCTGTCAC**GCC**AGTGGGT	3265–3246 (S → G)
VP1-225 forward	GTGAAT**GA**TGGGGGCCCCGGT	3115–3135 (G → D)
VP1-225 reverse	ACCGGGGCCCCC**AT**CATTCAC	3135–3115 (G → D)
VP1-258 forward	TGCCAATAT**CGA**AAAGCTGG	3210–3230 (E → R)
VP1-258 reverse	CCAGCTTTT**CG**ATATTGGCA	3230–3210 (E → R)
VP1-266 forward	GTGAATTTT**AA**ACCCACTAGC	3225–3255 (I → K)
VP1-266 reverse	GCTAGTGGG**TT**TAAAATTCAC	3255–3225 (I → K)
VP1-258/266 forward	CAATAT**CGA**AAAGCTGGCAATGTGAATTTT**AA**ACCCA	3214–3250 (E →R, I → K)
VP1-258/266 reverse	TGGG**TT**TAAAATTCACATTGCCAGCTTTT**CG**ATATTG	3250–3214 (E →R, I → K)
VP2-163 forward	CCACTGGG**CAG**ACAGCCGTGC	1430–1451 (E → Q)
VP2-163 reverse	GCACGGCTGT**CTG**CCCAGTGG	1451–1430 (E → Q)

Table 2. MAb reaction patterns against different SVDV strains and mutated viruses

Amino acids in pSVD146 were mutated to correspond to the relevant amino acids in isolate ITL/1/66. The location and result of these mutations are shown. The reaction pattern of MAbs used against different SVDV strains and mutated viruses in an ELISA is shown.

		MAbs				
Antigen	Mutation (aa)	143·9/10	145·2	145·12	5B7	HIS SVD*
SVD146		−	−	−	+	+
ITL/1/66		+	+	+	−	+
UKG/27/72		+	+	−	+	+
SPA/1/93		+	−	−	+	+
VP1 aa 87	G → D	+	−	−	+	+
VP1 aa 210	S → N	−	−	−	+	+
VP1 aa 225	G → D	−	+	−	+	+
VP1 aa 258	E → R	−	−	±	+	+
VP1 aa 266	I → K	−	−	±	+	+
VP1 aa 258, aa 266	E → R, I → K	−	−	+	+	+
VP1 aa 269	S → G	−	−	−	+	+
VP2 aa 163	E → Q	−	−	−	−	+

* A swine hyper-immune serum raised against UK/27/72 SVDV that reacts with all strains of SVDV.

both directions. Three amino acid (aa) substitutions compared to the parent strain sequence were found. At aa 449 in VP3, a methionine was changed to a threonine; at aa 1511 in 3A, a leucine was changed to a phenylalanine; and at aa 1776 in 3D, an aspartic acid was changed to asparagine.

Virus derived from pSVD146 was obtained by transfecting *Pvu*I-linearized pSVD146 into SK6T7 cells. In this cell line, plasmid DNA is transcribed by the stably expressed T7 polymerase. To increase virus titres, the supernatant of the transfected cells was passaged on IBRS-2 cells. The genome from the progeny virus, SVD146, was sequenced around the three amino acid substitutions that were different from the NET/1/92 parent strain. These substitutions appeared to be stable.

Epitope mapping and construction of antigenic variants of NET/1/92

The epitopes of MAbs that reacted specifically with strain ITL/1/66 but not with NET/1/92 were roughly mapped as described by Dekker *et al*. (2000). These results, obtained using a fusion-PCR technique, showed that MAbs 143·9 and 143·10 have their epitopes in the region between aa 5 and aa 87 of VP1, whereas the other MAbs used have their epitopes in the region between aa 199 and aa 283 of VP1. The fusion-PCR results, taken together with the reaction pattern of these MAbs with SVDV strains SPA/1/93, UKG/27/72, ITL/1/66 and NET/1/92 (Table 2) and the sequence data (Fig. 2), resulted in a few amino acids that could be a candidates for epitope formation. The amino acids possibly involved in epitope formation were aa 225 or aa 269 of VP1 for MAb 145·2. For MAb 145·12, aa 210, aa 258 or aa 266 of VP1 were implicated and for the MAbs 143·9 and 143·10, aa 87 of VP1 was implicated. In these experiments, the epitope for MAb 5B7, from which an essential amino acid was previously localized at aa 163 in VP2 (Nijhar *et al*., 1999), was used as a positive control.

The predicted amino acids were mutated in pSVD146 in such a way that they matched the relevant amino acids in the ITL/1/66 sequence. For these specific mutations, the primers used are described in Table 1. The viruses obtained after transfection of the mutated pSVD146 were tested by ELISA to investigate the reaction pattern of the MAbs. HIS SVD was used as a positive control to check for the presence of antigen.

As expected, the reaction of MAb 5B7 with SVD146 (mutated at aa 163 of VP2) became negative in the ELISA after the change of glutamic acid (pSVD146) to glutamine. Subsequently, all the above-described candidate amino acids were mutated in pSVD146 and the mutated viruses obtained were analysed by ELISA in order to see differences in MAb reaction patterns (Table 2). Two different amino acids involved in the epitope for MAb 145·2 were predicted. The amino acid at aa 225 of VP1 was changed from a glycine (pSVD146) to an asparagine or aa 269 of VP1 was changed from a serine (pSVD146) to a glycine. The change of aa 225 of VP1 showed a positive response in the ELISA; the other mutation did not change the reaction pattern of MAb 145·2 (Table 2). For MAbs 143·9 and 143·10, only aa 87 of VP1 was predicted to be important in epitope formation. Therefore, this amino acid was changed from a glycine (pSVD146) to an aspartic acid. This resulted in MAb recognition of the mutant virus. Three

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          1                                   32
NET/1/92  QGP PGGVTEGIIA RVADTVGSGP VNSESIPALT
ITL/1/66  QGP PGEAIERAIA RVADTIGSGP VNSESIPALT
UKG/27/72 QGP PGEVMGRAIA RVADTIGSGP VNSESIPALT
SPA/1/93  QGP PGGVTEGIIA RVADTVGSGP VNSESIPALT
          ------------------------------------
          33                                  82
NET/1/92  AAETGHTSQV VPSDTMQTRH VKNYHSRSES TVENFLCRSA CVFYTTYKNH
ITL/1/66  AAETGHTSQV VPSDIMQTRH VKNYHSRSES TVENFLCRSA CVFYTTYKNH
UKG/27/72 AAETGHTSQV VPSDTMQTRH VKNYHPRSES TVENFLCRSA CVFYTTYKNH
SPA/1/93  AAETGHTSQV VPSDTMQTRR VKNYHSRSES TVENFLCRSA CVFYTTYKNH
          ------------------------------------------------------
          83                                 132
NET/1/92  DSDGGNFAYW VINARQVAQL RRKLEMFTYA RFDLELTFVI TSTQEQSTTQ
ITL/1/66  DSDGDNFAYW VINTRQVAQL RRKLEMFTYA RFDLELTFVI TSTQEQSTIQ
UKG/27/72 DSDGDNFAYW VINTRQVAQL RRKLEMFTYA RFDLELTFVI TSTQEQPTVR
SPA/1/93  DSDGDNFAYW VINARQVAQL RRKLEMFTYA RFDLELTFVI TSTQEQSTTQ
          -----
          133                                182
NET/1/92  GQDTPVLTHQ IMYVPPGGPV PTKVNSYSWQ TSTNPSVFWT EGNAPPRMSI
ITL/1/66  GQDSPVLTHQ IMYVPPGGPV PTKVNSYSWQ TSTNPSVFWT EGSAPPRMSI
UKG/27/72 GQDAPVLTHQ IMYVPPGGPV PTKVNSYSWQ TSTNPSVFWT EGSAPPRMSI
SPA/1/93  GQDTPVLTHQ IMYVPPGGPV PTKVNSYSWQ TSTNPSVFWT EGNAPPRMSI

          183                                232
NET/1/92  PFIGIGNAYS MFYDGWARFD KQGTYGISTL NNMGTLYMRH VNGGGPGPIV
ITL/1/66  PFIGIGNAYS MFYDGWARFD KQGTYGINTL NNMGTLYMRH VNDGGPGPIV
UK/27/72  PFIGIGNAYS MFYDGWTRFD KQGTYGISTL NNMGTLYMRH VNDGGPGPIV
SPA/1/93  PFIGIGNAYS MFYDGWARFD KQGTYGISTV NNMGTLYIRH VNGGGPGPIV
                              ==================================
          233                                282
NET/1/92  STVRIYFKPK HVKTWVPRPP RLCQYEKAGN VNFIPTSVTE GRTDITTMKT T
ITL/1/66  STVRIYFKPK HVKTWVPRPP RLCQYRKAGN VNFKPTGVTE GRTDITTMKT T
UK/27/72  STVRIYFKPK HVKTWVPRPP RLCQYQKAGN VNFEPTGVTE GRTDITTMKT T
SPA/1/93  STVRIYFKPK HVKTWVPRPP RLCQYEKAGN VNFVPTSVTE GRTDITTMKT T
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Fig. 2. The consensus sequence of VP1 of NET/1/92 (matching the sequence of SVD146) compared to the sequences of SVDV strains SPA/1/93, ITL/1/66 and UKG/27/72 in the regions where epitopes were expected. Bold and underlined amino acids differ from the NET/1/92 consensus sequence. The predicted region (––) from fusion-PCR results in which MAbs 143·9 and 143·10 react is shown. The predicted region (= =) from fusion-PCR results in which MAbs 145·2 and 145·12 react is shown.

possible amino acid candidates (aa 258, aa 266 or aa 269 of VP1) were proposed for MAb 145·12. Neither of the mutated viruses resulted in a strong specific reaction with this MAb. The mutation of either aa 258 (absorbance value of 0·23) or aa 266 (absorbance value of 0·53) of VP1 in pSFV146 resulted in an intermediate response in the ELISA. Therefore a double mutation was performed at locations aa 258 and aa 266 of VP1. This resulted in a strong reaction in the ELISA of MAb 145·12 (absorbance value of 1·4). Amino acid substitutions and MAb reaction patterns are shown in Table 2.

Characteristics of SVD146 virus

Single-step growth curves of SVD146 on IBRS-2 and PK2 cells were performed and the results showed that the virus had the same growth properties as the parent strain NET/1/92 (Fig. 3). The plaque sizes of SVD146 and NET/1/92 were also similar (data not shown). Furthermore, the reaction pattern of a panel of MAbs raised against NET/1/92 was the same for both SVD146 and NET/1/92 (data not shown).

To study whether SVD146 was as virulent as NET/1/92, an *in vivo* study was performed. Both viruses were inoculated into the bulb of the heel of five 12-week-old pigs using a titre of 10^5 $TCID_{50}$/ml. In both groups, the first lesions were observed 2 days after infection. In the group infected with NET/1/92, four out of five pigs developed lesions; in the

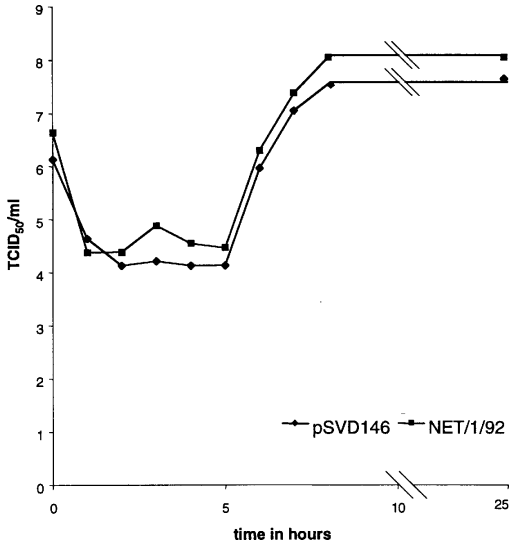

Fig. 3. Single-step growth curves of SVD146 and NET/1/92 on IBRS-2 cells.

group infected with SVD146, all pigs developed lesions. The lesion score during the experiment between both strains was comparable. In all pigs, seroconversion was observed by day 5 and lasted until the end of the experiment. The neutralization titre of both groups reached the highest level 12 days after infection (Table 3). Virus was isolated from faecal samples 5 days after infection from all pigs and the titres between the groups were comparable. Viraemia was detected in both groups. The results are summarized in Table 3.

Discussion

From the Dutch SVDV isolate NET/1/92, a full-length infectious cDNA clone, pSVD146, was successfully constructed. After transfection of the DNA into SK6T7 cells, infectious virus SVD146 was obtained. The *in vitro* characteristics, antigenicity, growth curve and plaques of the virus SVD146 were identical to the parent NET/1/92 virus. *In vivo* experiments also showed that the clinical disease characteristics between SVD146 and the parent strain were the same. Therefore, we concluded that pSVD146 is an accurate cDNA copy of the NET/1/92 strain genome. The three amino acid differences of the SVD146 genome compared to the NET/1/92 genome were stable and did not influence the growth and virulence properties of the SVD146 virus compared to the NET/1/92 virus. Also these amino acids are not involved in epitope formation as analysed with the MAbs available thus far.

All MAbs used in this study for epitope mapping could discriminate between isolates NET/1/92 and ITL/1/66 and are specific for ITL/1/66; the amino acids involved in these epitopes are located in VP1 (Dekker *et al.*, 2000). To map these epitopes in more detail, we have predicted which essential amino acids could be involved in the epitope formation and

Table 3. Experimental infection of pigs with SVD146 or NET/1/92

Virus	Dose ($TCID_{50}$)*	Mean lesion score (range)	Seroconversion	Highest neutralization titre (day 12)
NET/1/92	$10^{5·3}$	37·4 (0–56)	5/5	$4·1 \pm 0·4$
SVD146	$10^{5·5}$	34·6 (18–55)	5/5	$3·9 \pm 0·3$

* Expressed as log $TCID_{50}$/ml.

changed these individual amino acids in pSVD146 to the same amino acids as in strain ITL/1/66. To check whether this approach worked, we used MAb 5B7 for which the epitope (aa 163 of VP2) is known and which discriminates between isolates ITL/1/66 and NET/1/92 (Nijhar *et al.*, 1999). The reaction pattern of this MAb changed due to a single amino acid change. Other MAbs that are specific for the NET/1/92 isolate still reacted with the mutated virus, without a change in reactivity (data not shown). With this strategy it was shown that our approach could lead to the mapping of epitopes.

The epitope recognized by MAb 145.12 needed a double amino acid substitution in pSVD146 (aa 258 and aa 266 of VP1) for the formation of the epitope. To date, these amino acids, located at the C-terminal end of VP1, have not been described in epitope formation. This region is known for antigenicity in different picornaviruses (Minor *et al.*, 1986; Sherry & Rueckert, 1985). The double amino acid substitution that is necessary for the epitope of MAb 145·12 is probably due to the conformation needed for maximum recognition of this MAb, which is only correct when both amino acids are changed. Two other epitopes of SVDV were previously mapped to the C-terminal part of VP1, aa 272 of site 3A and aa 275 of VP1, which is homologous to site 3A of poliovirus (Kanno *et al.*, 1995). Another C-terminal epitope of SVDV VP1 is located at aa 261 (Nijhar *et al.*, 1999). The amino acid important for epitope formation used by MAb 145·12 described in this study is located in the same part of the C-terminal end of VP1. The epitope forming VP1 aa 225, recognized by MAb 145·2, has not been identified in SVDV before. The epitope is probably located in the βH–βI loop. Our results show that MAbs 143·9 and 143·10 recognized the same epitope located at aa 87 of VP1. This amino acid is located in the βB–βC loop, which is analogous to poliovirus antigenic site 1. The localization of this epitope has been described previously for the SVDV MAb 4G07 (Kanno *et al.*, 1995). The two new neutralizing antigenic sites described here are located in the ITL/1/66 strain, as the MAbs used are specific for ITL/1/66. The antigenic sites, aa 258 and aa 266, however, have been recently reported (Borrego *et al.*, 2000) and are also found in recently isolated Italian SVDV isolates. Thus, these epitopes are important antigenic sites, not only for the ITL/1/66 but also for other SVDV strains.

We describe two new amino acid positions important for epitope formations of SVDV, whereas another SVDV epitope described here was previously mapped by others. In conclusion a full-length infectious cDNA clone, pSVD146, of SVDV strain NET/1/92 was prepared. The derived virus had the same *in vitro* and *in vivo* properties as the parent strain. Therefore pSVD146 seems to be suited for studies in virulence, antigenicity and pathogenesis of this European SVDV isolate.

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