Persistent infection is a rare sequel following infection of pigs with swine vesicular disease virus

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SUMMARY

Nine isolates from pigs persistently infected with a recent Italian isolate of swine vesicular disease (SVD) virus, ITL/9/93, were collected sequentially over 121 days and were characterized antigenically and biochemically. There was an accumulation of amino acid (aa) substitutions in the capsid proteins throughout the carrier state that could be correlated with alterations in antigenicity in virus isolates collected late stage in infection. The aa substitutions detected mainly occurred in VP1 and antigenic changes were detected in late isolates both at antigenic site 1, resulting in loss of binding of Mab 4GO7, and at a closely located site which has not yet been named, recognized by Mab C29. In further experiments groups of pigs were exposed to a range of SVD viruses, but no virus was isolated beyond 16 days post infection (dpi) nor viral RNA detected beyond 42 dpi. Attempts to transfer infection to sentinel pigs introduced some time after initial infection of the original pigs were largely unsuccessful. The carrier state was established in only one out of five experimental infections of pigs with SVD virus and can therefore be considered a rare sequel to infection with SVD virus and is of limited significance in the epidemiology of the disease.

INTRODUCTION

Swine vesicular disease (SVD) is a notifiable disease of pigs included on the Office International des Épizooties (OIE) List A. The first outbreak of a vesicular, enterovirus disease of pigs later to be called SVD, was recognized in Italy in 1966 [2]. Subsequently the disease spread to other European countries [3, 4]. Although the disease was diagnosed in Hong Kong after 1966, it had probably been present in Asia before being detected in Europe. The disease is economically important as it is clinically indistinguishable from foot-and-mouth disease (FMD). It was eradicated from all countries of the European Union (EU) with the exception of Italy during the 1980s. However,

during the early 1990s, several member states of the EU suffered temporary incursions of the disease [4, 5], where it severely disrupted trade until all affected and in-contact pigs had been slaughtered. Outbreaks continue to occur in Italy where, in spite of an official eradication programme, it seems likely to persist for the foreseeable future. Currently no SVD vaccines are available. SVD is also present in Taiwan, Province of China and mainland China.

The genome of SVD virus consists of a single positive strand of messenger-active RNA comprising a 5′ untranslated sequence followed by an open reading fragment encoding the polyprotein precursors to the structural (PI) and the non structural (P2, P3) proteins, followed by a short non-coding sequence and finally a poly (A) tract at the 3′ terminus. The P1 protein is cleaved into the four structural proteins VP4 (1A), VP2 (1B), VP3 (1C) and VP1 (1D), of

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Table 1. *The agreement between changes of amino acid sequence and antigenicity in sequential isolates of SVDV*

Virus	Amino acid position in VP1*			Binding to C29 and 5B7 (%) in ELISA	
	80	85	91	C29	5B7
SVDV/ITL/9/93/P	K	D	Y	100	106
SS23/F/7/T	K	D	Y	> 89	98
SS23/F/121/T	K	G	H	< 18	100
SS27/F/28/T	K	D	Y	> 106	106
SS27/F/63/T	K	D	Y	98	105
SS27/F/121/D	K	G	H	na	na
SS27/F/121/T	K	G	Y	> 85	105
SS31/F/7/T	K	D	Y	> 92	98
SS31/F/121/T	K	G	H	< 4	> 98
SS36/F/7/T	K	D	Y	> 101	100
SS36/F/121/D	R	G	H	na	na
SS36/F/121/T	K	G	H	< 10	96

Nomenclature of virus isolates: pig number/origin/dpi/isolate; F, faeces; T, isolate passaged in tissue culture, D, isolate without passage in tissue culture; P, parental virus, *, Numbering according to SVDV/27/72 [11]; na, not available. ELISA reactivity as defined by Samuel et al. [13] was classified as very high (> 76%), high (46–75), medium (20–45%) or none (< 20%).

which 1B, 1C and 1D make up the surface of the virion, and includes the sites at which neutralizing antibodies can act.

Our previous study [1], together with the work of Gourreau et al. [6], showed that a persistent infection with SVD virus can occur; the carrier being defined as one from which live SVD virus can be recovered for more than 28 days after infection. The nucleotide sequences of the part of 1D gene from SVD virus isolates recovered from the later stage of infection, without passage in tissue culture, showed sequence differences from the parental SVDV/ITL/9/93 strain used to establish persistent infection [1]. It was not clear, however, whether or not the genomic, and consequent phenotypic, changes that occurred in the virus during persistence were sufficient to explain how the persistent virus evaded the host immune response. In addition, it was not clear whether or not persistent infection arose through selection of pre-existing variants in the 'quasi-species' of SVD virus that was used as the inoculum or whether genetic, and antigenic, variation arose due to mutation during infection.

It has been proposed that persistent infection results in the selection of variants better fitted to survive in the host [7, 8]. For picornaviruses, infection of mice with a mouse-adapted poliovirus mutant strain resulted in prolonged recovery of viral genome [9] and foot-and-mouth disease virus may persist in the pharynx of cattle for over 3 years following infection

[10], although the factors which allow this evasion of the immune system are not know. Similarly, for influenza virus Helten et al. [7] reported that experimental infection with a persistent influenza C virus variant led to prolonged genome detection in the chicken lung. Whether these observations are applicable to SVD virus remains uncertain but our previous studies [1] suggested that SVDV may persist by evading the host immune response. Moreover, it is not known if persistent infection of SVDV is a common consequence of infection and therefore of epidemiological significance.

In the study reported here, sequential isolates recovered from the previous successful persistence experiment have been antigenically characterized using monoclonal and polyclonal sera and partially sequenced. Four further experiments are described in which groups of pigs were exposed to a range of SVD virus isolates and the frequency of the carrier state investigated.

MATERIALS AND METHODS

Antigenic and genomic characterization of SVD virus isolates recovered from a persistent infection [1]

SVD virus isolates used for characterization

The SVD virus persistent isolates from the previous persistence experiment [1] which were further characterized are listed in Table 1. The consecutive isolates

Table 2. *Mabs used for antigenic profiling*

Mab	Parental virus	Binding site	Residues (aa) of VP	Working dilution
4G07	H3′76*	Site 1	VP1, 87, 88	1:100 (NI)
C29	UKG/27/72†	Site?	VP1, 261	1:500 (ELISA)
5B7	ITL/72‡	Site 2a	VP2, 163	1:200 (ELISA), 1:100 (NI)
6H07	H3′76	Site 2b	VP2, 154	1:100 (NI)
30B09	H3′76	Site 3a	VP1, 272, 275; VP3, 60	1:100 (NI)
23F10	H3′76	Site 3b	VP2, 70, 233; VP3, 76	1:100 (NI)

A panel of six Mabs raised against three different strains of SVDV were used in the studies with the properties shown. Binding sites were determined by examining the nucleotide sequence of Mab escape mutants [14, 15].
* According to Kanno et al. [14]
† WRL Reference.
‡ According to Brocchi et al. [16]
The four Mabs raised against SVDV/H3′76 were kindly provided by Dr T. Kanno.

Table 3. *Oligonucleotide primers used for RT, PCR and sequencing*

Olionucleotide designation	Sequence (5′–3′)	Position on the SVDV genome*		Used for
Kanno-1 (sense)†	TTAAAACAGCCTGTGGGTTGTT CCCACCCA	5′-NCR	1–30	PCR
LSVDV/871F (sense)	ACAAGACTTCACACAGGACC	1A	871–890	Sequencing
LSVDV/1232F (sense)	AACATGCAGTACCACTACCT	1B	1232–1251	Sequencing
LSVDV/1591F (sense)	CAGGCACAACAATTTTACAC	1B	1591–1610	Sequencing
LSVDV/1961F (sense)	ACCAACGGTTCCCAAGTCTTTG	1C	1961–1982	Sequencing
pSVD-1C575 (sense)	GCTGGTATCAAACAAATATT GTGG	1C	2307–2330	Sequencing
GSVD-3 (sense)	ACACCCTTTATAAAACAGG	1C	2414–2432	PCR+ sequencing
LSVD-1 (sense)	TTCTTTCAAGGGCCCCCAGGAG	1C/1D	2434–2455	PCR+ sequencing
LSVDV/2901F (sense)	TGCCCACAAAGGTGAACAGT	1D	2901–2920	Sequencing
LSVD-1D496F (sense)	AACCGGAGTGTGTTCTGGAC	1D	2942–21961	Sequencing
GSVD-5 (antisense)	AACATGCTGTATGCGTTG CCTAT	1D	3005–3027	PCR+ sequencing
LSVDV/317F (sense)	AGCATGTCAAAACATGGGTC	1D	3171–3190	Sequencing
NK-45 (antisense)	GCCAACGTACACGGCACC	2A	3317–3334	RT+PCR+ sequencing

* Numbering according to SVDV/UKG/27/72 [12]; † Kanno T (unpublished data).

of SVD virus were passaged three times on IB-RS-2 cells [11] to achieve a titre suitable for antigenic profiling and sequencing. After the final passage, the culture supernatant was clarified by centrifiguation at 1000 *g* for 10 min, mixed with an equal volume of glycerol, and stored at $-20°$ C.

Monoclonal and polyclonal antibodies

The isolates listed in Table 1 were antigenically characterized using a panel of six monoclonal antibodies (Mab) raised against three different strains of SVDV (Table 2). Polyclonal rabbit and guinea-pig antisera to SVDV/ITL/9/93 were kindly provided by Dr N. Ferris, IAH, UK.

Oligonucleotide primers used for RT, PCR and sequencing

The sequences and locations of oligonucleotide primers used for reverse transcription–polymerase chain reaction (RT–PCR) and for sequencing the 1B, 1C and 1D genes of sequential isolates are listed in Table 3.

Mab profiling ELISA

Selected virus isolates collected early or late in the course of infection were antigenically characterized in an indirect antigen-trapping enzyme-linked immunosorbent assay (ELISA) developed for differentiating

foot-and-mouth disease virus isolates by Samuel et al. [13] using Mabs 4G07, 5B7, 6H07, 30B09, 23F10 and C29. The ELISA was repeated three times to ensure the consistency of the result.

Virus neutralization index test

The persistent SVD virus isolates were compared using the virus neutralization index (NI) test [15] and a panel of six Mabs in a 96 well microplate (Nunc, Maxisorb, Denmark). In brief, 50 μl of twofold dilutions of virus and 50 μl of 1/100 dilutions of Mabs were mixed and incubated at 37 °C for 1 h. Fifty μl of IB-RS-2 cells (300 000 cells) in Eagle's MEM was then added and the plates were incubated in a CO_2 incubator for 2 days at 37 °C. The plates were then read microscopically for the presence of cytopathic effect (CPE). The 50 % end-point titre of the virus with and without Mab was calculated according to the method of Kärber [18]. The test was repeated three times to ensure reproducibility of results and the NI of each Mab was expressed as the difference between the titre of the virus alone and that of the virus-Mab mixture. A NI of 0·6 Log_{10} was the minimum value considered to be significant.

RT–PCR

Total RNA was prepared from cell culture fluid using the Qiagen RNeasy Mini kit (Qiagen Ltd, UK). RT was performed with primer NK 45 and M-MLV reverse transcriptase, and RNase H minus according to manufacturers instructions, with the exception that 9 μl of total RNA used as template (Gibco–BRL, UK).

For the long PCR, two separate master mixes were prepared; master mix one containing 1·75 μl of dNTPs, 1 μl of primer NK 45, 1 μl of primer Kanno-1, 5 μl of SVDV cDNA from the above reaction mixture and 16 μl of DEPC treated H_2O; master mix two containing 5 μl of 10 × Expand™ Long Template PCR System buffer, 0·75 μl of enzyme mix (Taq and Pwo DNA polymerase, Boehringer–Mannheim Ltd, UK). The two master mixes were mixed together and overlayed with 30 μl of mineral oil. The reaction mix was incubated in a thermal cycler (Omnigene, Hybaid Ltd, UK) at 94 °C for 2 min followed by 10 cycles of the following temperature and time: 94 °C for 10 s, 65 °C for 30 s and 68 °C for 4 min; then 6 cycles of 94 °C for 10 s, 65 °C for 30 s and 68 °C for 4 min 20 to 6 min (adding a cycle elongation time of 20 s for each cycle, e.g. cycle No. 11 has an additional 20 s and

cycle No. 12 has an additional 40 s); then 14 cycles of 94 °C for 10 s, 65 °C for 30 s and 68 °C for 6 min 20 s; and the final extension was at 68 °C for 7 min.

The expected sizes of PCR-amplified fragments with the primer pair NK 45/Kanno-1 was 3334 bp.

The detection of SVD vRNA using RT-nPCR in faeces

The total RNA was extracted from suspensions of faeces and SVD vRNA was detected by RT-nested PCR (RT-nPCR), as described by Lin and colleagues [19]. The RT-nPCR amplified a fragment of 594 base pairs in length corresponding to part of the capsid-coding region (1C and 1D) of the SVDV genome.

Capsid sequencing

Capsid sequencing was performed by direct cyclic sequencing using the *fmol*[7] cyclic sequencing kit (Promega, UK). Every base of the cDNA was covered at least twice using the primers listed in the Table 3.

Duration and transmission of infection with different SVD virus isolates

Viruses

SVD virus isolate ITL/9/93 was isolated in 1993 from an outbreak of clinical disease in Forli, Italy. The original isolate was passaged four times on IB-RS-2 cells to achieve a titre suitable for experimental inoculation. Plaque-purified SVD virus ITL/9/93 was derived from a large plaque phenotype of SVDV/ITL/9/93 and plaque-purified three times on IB-RS-2 cells. Consequently, the plaque-purified virus was passaged a total of nine times on IB-RS-2 cells. The SVD virus persistent isolate SS23/F/121/T recovered from the carrier pig SS23 in the previous persistence experiment [5] was passaged three times on IB-RS-2 cells. SVD virus isolate ITL/11/98 was isolated in 1998 from an outbreak of clinical disease in Lonato, Italy and passaged three times on IB-RS-2 cells.

For all viruses, after the final passage the culture supernatant was clarified by centrifugation at 1000 *g* for 10 min, and stored at −20 °C. The titre of the inoculum was determined by end-point titration on IB-RS-2 cells before use.

Experimental animals

All pigs used in the study were 6-week-old Landrace cross-bred large white pigs of approximately 20 kg in

weight. They were all housed in the disease secure facility at IAH, Pirbright.

Animal experimental protocols

Experiment 1: Pigs infected with plaque-purified SVDV/ITL/9/93

Eighteen pigs were divided into two groups: one experimental group of 14 pigs (TD80–TD93) and one negative control group of 4 pigs (TD94–DT97) which were later introduced to the experimental group. On the first day of the experiment (day 0) all 14 experimental (TD80–TD93) were inoculated with $10^{6.5}$ $TCID_{50}$ of plaque-purified SVDV/ITL/9/93 in 1 ml of tissue culture fluid by intradermal inoculation into the bulb of the heel of the right fore leg. Rectal temperatures were measured daily for 10 days, and pigs were examined for clinical signs at 3, 5, 7, 10, 14 and 21 dpi. At 28 and 56 dpi the pigs were washed, sprayed with disinfectant (FAM, Evans Vanodyne, UK), and rinsed with water to remove any contaminating virus. They were then moved to a new, clean pen into which was also introduced two new, seronegative pigs from the negative control group. For the first 7 days after mixing, faeces were allowed to accumulate in the pen and there was minimal cleaning to ensure a high level of potential exposure to SVD virus in the in-contact animals (this procedure was also used in the following experiments after introducing clean pigs). The newly introduced pigs were examined for clinical signs, and their rectal temperature was measured, for the first 14 days after introduction. Each pair of newly introduced pigs was killed at 21 days after first exposure to the infected pigs.

Two pigs from the original experimental group were killed at 7, 28 and 56 dpi to examine tissues for the presence of virus and viral RNA post mortem. The experiment was terminated at 85 dpi.

Experiment 2: Pigs infected with persistent isolate SS23/F/121/T

Eighteen pigs were divided into two groups: one experimental group of 8 pigs (TG86–TG93); and one negative control group of 10 pigs (TG94–TG97, T126, TG127, T199, TK00, TK56 and TK57) which was later introduced to the experimental group.

On the first day of the experiment, 6 (TG86–TG91, infected pigs) out of the 8 pigs in the experimental group were inoculated with $10^{6.2}TCID_{50}$ of the persistent isolate SS23/F/121/T in 1 ml of tissue culture fluid as described in Experiment 1. The two remaining pigs (TG92 and TG93, exposed pigs) in the experimental group were kept in contact with the directly-inoculated animals for the duration of the experiment. For the first 7 days after inoculation, faeces were allowed to accumulate in the pen and there was minimal cleaning to ensure a high level of exposure to SVDV in the in-contact animals. Following inoculation, measurement of rectal temperature and examination for clinical signs were carried out as described in Experiment 1. At 14 dpi, all pigs in the experimental group were washed, sprayed with FAM, rinsed with water and moved into a new pen. At the same time, two washed pigs (TG94 and TG95) from the negative control group were introduced into the same pen to form a new group. This procedure of introducing two clean pigs to the infected group was repeated monthly starting at 28 dpi for a total of four times. The same procedure for examining and killing the newly introduced pigs was carried out as described for Experiment 1 above. All eight experimental pigs were killed at 133 dpi.

Experiment 3: Pigs infected with SVDV/ITL/9/93

Twenty-six pigs were divided into 3 groups: 2 experimental groups, each of 12 pigs (Group 1: pigs TM55–TM66; Group 2: pigs TM67–TM78), and a negative control group of 2 pigs (TM53 and 54). This experiment and Experiment 4 reproduced the protocol of the previous successful experiment [1] in which 2 pigs in each group of 12 were inoculated with $10^{7.4}TCID_{50}$ of SVDV virus ITL9/93 into the heal bulb and left in contact with the remaining 10 pigs. For the first 7 days after inoculation minimal cleaning took place in the boxes, to allow high exposure of the uninoculated pigs to virus. All animals were clinically examined on days 3, 5, 7, 12, 14 and 21 and regularly thereafter. The experiment was terminated at 121 dpi.

Experiment 4: Pigs infected with SVDV/ITL/11/98

Twenty-six pigs were divided into 3 groups: 2 experimental groups, each of 12 pigs (Group 1: TV1–12; Group 2: pigs TV13–24), and a negative control group of 2 pigs. The experimental protocol was carried out as in Experiment 3 and the previously reported experiment [1], but to increase the virus load, in each of the 2 groups of 12 pigs, 4 were directly inoculated with $10^{7.4}$ $TCID_{50}$ of SVDV/ITL/11/98 in 1 ml of tissue culture fluid and the remainder were

exposed to infection by contact. At 70 dpi, 28 days after the last detection of viral RNA in faecal samples, the two groups of pigs were washed, sprayed with FAM, rinsed with water and mixed by moving both to a clean box and two new pigs were introduced. All pigs were clinically examined for a further 2 weeks. The experiment was terminated at 84 dpi.

In all four experiments blood and faecal samples were collected regularly for the detection of antibody to SVDV and for the examination of the presence of virus and viral RNA respectively. Measurement of total antibody of SVDV was carried out by the virus neutralization test (VNT) using SVDV/ITL/9/93 as antigen in the micro-neutralization assay as described by Golding et al. [20]. Virus isolation and the detection of viral RNA in faecal samples were performed according to methods described by Lin et al. [1, 19].

RESULTS

Characterization of persistent SVD virus isolates

Mab profiling ELISA

Of the six Mabs in the panel, two (C29 and 5B7) reacted with the infecting isolate SVDV/ITL/9/93 in the strain characterization ELISA (Table 1). Mab C29 showed a loss of binding to most virus isolates recovered late during the course of infection as compared with the infecting virus and early isolates. An interesting exception was late isolate SS27/F/121/T which was recognized by Mab C29, despite being isolated late in the course of infection. Mab 5B7 bound the infecting virus, early isolates and late isolates equally well. The remaining four Mabs reacted only poorly in ELISA with either the infecting virus or with recovered viruses.

Virus neutralization index

Mab C29 neutralised the parental virus and early isolates equally well but most of the late isolates escaped neutralization. In the NI test, as in ELISA, the late isolate SS27/F/121/T was an exception in that it was neutralized by Mab C29. Mab 4G07 neutralized the parental virus and early isolates, but did not neutralize any of the three late isolates examined. In contrast, Mabs 5B7 and 23F10 neutralized parental virus and all sequential isolates to a high titre. Mabs 6H07 and 30B09 did not neutralize either the infecting virus or any sequential isolates, suggesting that antigenic sites of 2b and 3a of the

Japanese SVDV isolate H3′76 are not present on SVDV/ITL/9/93 (Table 4).

Genomic variation in sequential isolates from persistently infected pigs

Nine SVD virus isolates were used for determining the nucleotide sequence of the 1B, 1C and 1D genes (Fig. 1). Sequencing of 1B, 1C and 1D genes showed that the SVD virus genome did not undergo extensive mutations during persistence, but that there was a limited accumulation of nucleotide substitutions over time. Sequence changes were not detected in samples collected at 7 dpi, but by 63 dpi a maximum of 12 nucleotide substitutions were detected in isolate SS27/F/63/T recovered from faeces. However, none of these nucleotide changes was conserved in viruses isolated at 121 dpi, but up to four base changes were detected at other sites (Fig. 1). When the consensus sequence of the parental virus (SVDV/ITL/9/93) was compared with each consecutive isolate, the extent of variation ranged from 0·0% (e.g. SS36/F/7/T) to 0·5% (e.g. SS27/F/63/T). Nucleotide substitutions were not conserved between isolates at different times, but similar nucleotide changes were observed among isolates recovered at the same sampling time.

In general, the nucleotide sequence of the regions of the 1D gene derived by direct sequencing of RT-nested PCR amplicons from faecal samples (see sequences in [1]) showed a similar pattern of variation to sequences obtained from isolates passaged in tissue culture (Fig. 1). However, some differences, probably as a result of passage in tissue culture were detected. For example, SS27/F/121/D had cytosine at position 2717, whereas isolate SS27/F/121/T had thymine at this site. Likewise, SS36/F/121/D had guanidine at position 2685, whereas SS36/F/121/T had adenine. In all cases where differences were observed, viruses amplified in tissue culture showed reversions back to the parental sequence suggesting that the mutations detected by direct sequencing of persistent isolates were not stable *in vitro*.

Amino acid sequence variation in sequential isolates from persistently infected pigs

Examination of aa sequences, as deduced from nucleotide sequence of consecutive isolates, indicated that there was an accumulation of aa substitutions throughout the carrier state (Fig. 2). Although the largest number of nucleotide substitutions occurred in a virus isolate recovered at 63 dpi (SS27/F/63/T),

Table 4. *Characterization of persistent of SVDV isolates using the virus neutralization index test*

	4G07	C29	5B7	6H07	30B09	23F10
SVDV/ITL/9/93/P	1·6	> 1·60	> 1·60	0	0	> 1·60
SVDV/SS23/F/7/T	1·2	> 1·50	> 1·50	**0·15**	**0·15**	> 1·50
SVDV/SS23/F/121/T	**0·15**	**0**	> 1·05	**0**	**0**	> 1·05
SVDV/SS27/F/28/T	> 1·20	> 1·20	> 1·20	**0**	**0·3**	> 1·20
SVDV/SS27/F/121/T	**0·15**	> 0·75	> 0·90	**0·3**	**0**	> 0·90
SVDV/SS31/F/7/T	> 1·95	> 1·95	> 1·95	**0·15**	**0·15**	> 1·95
SVDV/SS31/F/121/T	**0**	**0**	> 1·60	**0**	**0**	> 1·60
SVDV/SS36/F/7/T	> 1·20	> 1·20	> 1·20	**0**	**0·3**	> 1·20
SVDV/SS36/F/121/T	**0·15**	**0**	> 1·35	**0**	**0**	> 1·35

Neutralization index (NI) of Mabs with consecutive isolates recovered from pigs infected with SVDV/ITL/9/93. NIs less than 0·6 indicate escape from neutralization and are shown in bold.

Fig. 1. The nucleotide sequences of 1B, 1C and 1D genes derived from the parental virus (SVDV/ITL/9/93) and sequential virus isolates recovered from pigs infected with SVDV/ITL/9/93. Differences in sequence between the parental virus and virus isolates derived from infected animals are shown. (˙) same as sequence of parental virus. The nomenclature used for viruses was as follows: Pig number/origin/dpi/tissue culture isolate; F, faeces; T, virus isolates passaged in tissue culture; P, parental virus. *Numbering according to SVDV/UKG/27/72 [12].

Fig. 2. Comparison of the amino acid sequences of VP2, VP3 and VP1 proteins deduced from the corresponding nucleotide sequences shown in Fig. 1. Only amino acids which differ from those of the parental virus are indicated. *Numbering according to SVDV/UKG/27/72 [12].

most of these were silent. In contrast, all four nucleotide changes detected in isolate SS31/F/121/T recovered at 121 dpi resulted in aa substitutions and three other isolates collected at 121 dpi also had the same substitutions at three out of these four sites (SS23/F/121/T, SS27/F/121/D and SS36/F/121/T). The extent of variation in aa sequence (VP1, VP2 and VP3) therefore varied from 0·0% (e.g. SS23/F/7/T, SS31/F/7/T and SS36/F/7/T) to 0·5% (SS31/F/121/T). Although all three externally exposed capsid proteins of SVDV contain regions which make up neutralizing sites [4], no aa substitutions were detected in VP2 of viruses collected in the late stages of infection. Most substitutions were in VP1 and only one was in VP3.

The three-dimensional (3D) structure of SVDV has not yet been resolved. It is likely that the structure of Coxsackievirus B3 (CVB3) virion is very similar in overall topology to that of SVDV as in order to align the capsid polypeptides of CVB3 and SVDV only two insertions are required. The four aa substitutions detected in VP1 and VP3 of viruses collected late in the course of infection were therefore mapped onto the 3D structure of CVB3 [21]. Of these substitutions,

Table 5. *Outcomes of infections with a range of SVD virus isolates in pigs*

Experiment No.	Virus	Infected pig no.	Exposed pig no.	Clinical no.*	Subclinical no. †	Last day VI+	Last day PCR+	Transmission to in-contacts
1	ITL 9/93 (cloned)	14	0	12	2	7	35	None at 28 and 56 dpi
2	ITL 9/93 (persistent)	6	2	7	1	16	28	Virus transmission at 14 dpi, none at 28, 56, or 84 dpi
3	ITL/9/93	4	20	10	14	7	21	None at 99 dpi
4	ITL/11/98	8	16	21	3	16	42	None at 70 dpi

VI+, Virus isolation positive; PCR+, RT-nested PCR positive [1].
* Number of pigs which developed clinical disease.
† Number of pigs which seroconverted without showing clinical disease.

Table 6. *Transmission of infection to in-contacts in Experiment 2*

Pig no.	dpi	Day post introduction			
		2	7	14	21
TG94	14	v	+	+ +	v
TG95	14	+ v	+ v	−	−
TG96	28	−	nd	−	− n
TG97	28	−	−	−	nd
T126	56	−	nd	−	− n
T127	56	−	−	−	− n
T199	84	−	nd	nd	nd
TK00	84	− n	nd	nd	nd
TK56	112	− n	−	nd	nd
TK57	112	− n	−	nd	nd

v, Virus isolation positive, n, virus isolation negative; +, RT-nPCR positive; −, RT-nPCR negative; nd, not done.

two (positions 80 and 85 of VP1) were located in, or close to, the BC loop region of VP1 that constitutes antigenic site 1 in SVDV [14]. Isolates with aa substitutions at residue 85 of VP1 were not neutralized by the Mab 4G07 which recognizes antigenic site 1 of SVDV (Tables 1, 4). The significance of a change at position 80, as seen by direct sequencing the late isolate SS36/F/121/D [1], is unclear as the sequence reverted to that of the parental strain on passage in tissue culture (Table 1, Fig. 2). The third substitution (position 91 of VP1) is predicted to lie in the β-sheet C and is located close to position 261 which has been found to be critical for the binding of neutralizing Mab C29 [13]. Mab C29 showed no binding with, and did not neutralize, virus isolates having substitutions at position 91 in ELISA and NI test (Tables 1, 4), respectively. Association between binding of Mab C29 and position 91 was further confirmed by the observation already made that reversion of histidine

(H) to the parental tyrosine (Y), as was observed following passage of isolate SS27/F/121/D in tissue culture, resulted in recovery of binding and neutralization by Mab CD29. The observation that changes at position 91 of VP1 did not affect the reactivity of Mab 4G07 with antigenic site 1 of SVDV, suggests that residues 91 and 261, with which Mab C29 interacts, form either a separate epitope within site 1 or constitute a separate site entirely. The final aa substitution identified in all four late isolates was a change at position 222 of VP3. This position is unlikely to affect antigenicity as it was not exposed on the outer surface of the virion. Other aa changes were detected in isolates recovered at varying times after infection. These were either not located close to any known sites of antigenicity (e.g. position 266 of VP1 of SS27/F/28/T, position 23 of VP1 of isolate SS31/F/121/T) or were internally located (e.g. positions 214 and 258 of VP1 of isolate SS27/F/63/T).

Duration and transmission of infection with different SVD virus isolates

The results of the four infection experiments carried out in the study are summarized in Table 5. All but two inoculated pigs developed clinical disease. The two exceptions were pigs inoculated with the cloned isolate of ILT/9/93 in Experiment 1 and these were sub-clinically infected as determined by seroconversion in the absence of clinical signs. Both the cloned and the persistent isolates were less virulent than the parental ITL/9/93 isolate (data not presented). The more recent isolate ITL/11/98 was, in turn, more virulent than ITL/9/93. A varying proportion of the pigs exposed to the inoculated pigs

showed clinical disease. Generally, the higher the clinical score of those pigs that did show disease, the greater the proportion of clinically affected in-contacts.

All clinically affected pigs seroconverted to SVD virus and remained positive at high titre until slaughtered. Serum antibody responses in sub-clinically infected pigs were generally much lower and many sub-clinically infected pigs became 'sero-negative' within 1 or 2 months after infection.

In none of these four experiments was virus isolated beyond 16 dpi or viral RNA detected beyond 42 dpi. The strain of virus used for infection did not affect the length of time for which pigs were infected. In all experiments viral RNA was detected for longer than virus could be detected in faeces.

Attempts to transfer infection to sentinel pigs introduced some time after initial infection of the original pigs were largely unsuccessful. None of the newly introduced pigs developed clinical signs or pyrexia. In only one instance, when the sentinels were introduced at 14 dpi in Experiment 2, was virus isolated from the newly introduced pigs (Tables 5, 6). Even in this case, it is not clear whether the pigs became actively infected or whether there was passive transfer of virus through the gut, as seroconversion did not occur even though virus was isolated for up to 7 days after introduction. Introduction of pigs subsequently did not result in seroconversion and neither virus nor viral RNA could be detected. When the sentinels were introduced at 28 dpi in Experiments 1 and 2, viral RNA could still be detected in some of the pigs by PCR. The fact that virus transmission did not occur from PCR-positive pigs to susceptible animals placed in-contact indicates that the risk of spread appears to be low from pigs which are PCR positive but from which virus can no longer be isolated.

DISCUSSION

For the establishment of the carrier state, antigenic variation may be one of the most important mechanisms by which persistent viruses evade the immune response of the host [22]. In this study Mabs identified antigenic changes both at antigenic site 1, resulting in loss of binding of Mab 4G07, and at a closely located site which has not yet been named, recognized by Mab C29. These two sites, though close on the aa chain, appear to be distinct sites or distinct epitopes within a single site. Site 1 is thought to be a

major antigenic site in SVD virus [14]. Using Mabs, Gebauer et al. [23] showed that the selection of variants during the carrier state in FMD in cattle occurs at the major antigenic site on VP1. Gebauer et al. [23] also demonstrated multiple point mutations within the VP1 coding regions which resulted in some amino acid substitutions in the capsid proteins. They proposed that aa substitutions in this protein could favour the survival of mutants and contribute to the escape of viral sub-populations from immune clearance mechanisms. However, this is still controversial, and no definitive explanation for FMD virus persistence is available [10]. Protein VP1 of SVD virus is responsible for the induction of neutralizing antibodies in infected pigs [24] and the majority of aa substitutions observed in the late isolates in the study reported here were in VP1. Therefore, the finding that Mabs 4G07 and C29 showed no reactivity with viruses having substitutions at position 85 and 91 of VP1, respectively, suggests that alterations in these two antigenic sites might be linked in some way to an ability to persist in the host. However, in our previous report [1] we showed that SVD virus isolates recovered from the late stage of infection were neutralized equally well by polyclonal sera collected either early or late in the course of infection. The antigenic changes between early and late isolates detected in this study must therefore be subtle and do not directly influence the extent to which the virus is neutralized in the host.

SVD virus, in common with other picornaviruses, has a great potential for mutation. The study reported here showed that there was a limited accumulation of aa substitutions with time throughout the course of infection. None of the aa substitutions detected in early isolates was conserved in viruses isolated late in the course of infection. However, similar substitutions were observed between isolates recovered at the same sampling time. These observations could be explained either by transient aa substitutions which did not become fixed within the genome or by the coexistence of heterogeneous populations in which variants evolved independently from each other, and predominated at irregular time intervals.

The establishment of persistent infection with SVDV might be related to selection within the host of a sub-population of virus adapted to survive *in vivo*. In poliovirus, establishment of persistent infection *in vitro* has been shown to rely on selection of virus with genetic mutations [25]. Failure to establish persistence in Experiment 1 might therefore be attributed to the

selection by plaque purification of a sub-population of viruses which was incapable of establishing the carrier state. Conversely, in Experiment 2, the sub-population with genetic and antigenic mutations used to infect pigs was an isolate of virus recovered from a pig in the carrier state. This isolate might be expected therefore to be more 'fit' for establishing persistent infection. Failure of the 'carrier' virus to result in persistent infection could indicate that the population of viruses required to establish the carrier is different from that required to maintain it. In fact, the carrier state was established in only one out of five experimental infections of pigs with SVD virus. Even an attempt to reproduce as closely as possible the protocol of the previously successful experiment failed to reproduce the persistent state (Experiment 3). These findings are consistent with the majority of published reports which found that SVD virus did not persist in pigs beyond 2–3 weeks after infection. The carrier state is therefore a rare sequel to infection with SVD virus.

The rare nature of the carrier state made it difficult to study the factors which permit the establishment and maintenance of persistent infection. It is likely that persistence is established when there is a combination of specific factors present in both the host and the virus and that having the required factors in only one is not sufficient.

No firm conclusions can be drawn as to the ability of persistently infected pigs to transmit infection to susceptible animals placed in-contact. Transmission of infection, but not disease, was seen only when pigs were introduced into a group of previously infected pigs from which it was still possible to isolate virus (Experiment 2) and, even in this case, true subclinical infection was not established as seroconversion did not occur. This is potentially significant, as such pigs would be missed if surveillance relied on serology alone. Although it was not possible in the current experiments to transmit clinical disease through exposure to infected faeces, the possibility of transmission remains, as exposure to a contaminated environment can be at least as infectious as exposure to infected pigs [26].

Transmission did not take place when pigs were introduced to pigs which were PCR positive in the absence of virus isolation (Experiments 1 and 2). The rarity of the carrier event and the failure to transmit from PCR positive pigs both suggest that transmission from carriers is likely to be infrequent. Care must also be taken when interpreting the results of PCR assays as the results suggest that viral RNA may be recovered for some time after replicating virus has ceased to be produced.

In conclusion, although changes in amino acid sequence, with corresponding changes in antigenicity, were detected in persistent isolates recovered from carriers, the significance of these changes remains unclear. The occasional discovery of persistence following experimental infection and the anecdotal reports of persistent infection in the field support the existence of the carrier state. The results of this study are consistent with these previous reports, indicating that the carrier state is a rare sequel to infection with SVD virus and is likely to be of limited significance in the epidemiology of the disease.

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