Epidemiol. Infect. (1998), **121**, 459–472. Printed in the United Kingdom

The persistence of swine vesicular disease virus infection in pigs

F. LIN, D. K. J. MACKAY* AND N. J. KNOWLES

Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, UK

(*Accepted 8 April 1998*)

SUMMARY

Two groups of pigs were infected with a recent Italian isolate of swine vesicular disease virus (SVDV). Blood, nasal swabs and faeces were collected for up to 6 months after exposure to infection and animals were killed at regular intervals to obtain tissues *post-mortem*. These samples were examined for virus by conventional means and for viral RNA (vRNA) by reverse transcription-nested polymerase chain reaction (RT-nPCR). Virus was identified intermittently from both clinically and subclinically infected animals in nasal swabs, faeces and tonsillar tissue by either virus isolation or RT-nPCR up to 63 days post infection (dpi). Between 63 and 119 dpi virus was not detected in the secretions, excretions or tissues of any pigs. Following mixing of the two groups of animals at 119 dpi, SVDV was again identified in faeces for up to 7 days suggesting that the stress of mixing reactivated the excretion of virus in pigs from which the agent could no longer be identified. Minor antigenic changes were identified between the parental virus and isolates recovered late in the course of infection. Altered antigenicity corresponded with deduced amino acid substitutions identified from differences in nucleotide sequence between early and late isolates. This investigation demonstrates that SVDV and vRNA can be present in pigs for considerably longer after exposure to infection than has previously been recognized and provides preliminary evidence for a carrier state in swine vesicular disease.

INTRODUCTION

Swine vesicular disease (SVD) is a viral disease of pigs characterized by the appearance of vesicular lesions on the limbs, around the mouth, and occasionally on the snout of affected animals. Although the disease is frequently mild in nature, it is important because of the similarity of the lesions to those produced by foot-and-mouth disease (FMD). The first outbreak of SVD was recognized in Italy in 1966 [1]. The disease occurred in Hong Kong in 1970 [2] and in the UK in 1972 [3]. Subsequent outbreaks were seen in a number of European and Asian countries [4]. Since 1992 there has been an increase in the number of outbreaks of SVD within the European Union [5, 6] and an SVD eradication campaign is currently underway in Italy.

Swine vesicular disease virus is a subspecies of coxsackievirus B5 and a member of the enterovirus genus in the family *Picornaviridae* [7, 8]. SVD virus infection can occur by a variety of routes, the most sensitive being through damaged skin [4]. Dekker and colleagues [9] demonstrated that contact with an environment contaminated with SVDV was as infectious as direct inoculation or contact with SVDV-infected pigs. After multiplication at the initial site of infection, the virus spreads and large amounts are found in the secretions and excretions of the pig

* Author for correspondence.

before the appearance of lesions [10]. At the same time as the lesions appear, large quantities of virus are present in vesicular material and in the tissues of the body. The amount of virus detected decreases once antibody starts to be produced. Subclinical infection, characterized by seroconversion and viral excretion without clinical signs, is recognized [11, 12]. Using conventional techniques for virus isolation, most studies to date suggest that SVDV is rapidly eliminated from infected pigs. The virus does not generally persist in the tissues longer than 14 days and cannot be recovered from oral or nasal swabs beyond 7 dpi [13]. Excretion in faeces usually occurs for up to 23–39 dpi [11, 12]. A study in France [14] reported that virus was recovered in pharyngeal mucus up to 90 dpi, nasal mucus up to 80 dpi, urine up to 90 dpi and faeces up to 100 dpi following infection with the virulent French isolate 2862 L.C.R.V.. However, the ability of these pigs to transmit disease or infection was not investigated. Both historically in the UK [15, 16], and more recently within Europe [17], outbreaks of SVD have occurred which could not be traced back to any known source. Previous attempts to identify the carrier state in SVD have been unsuccessful [13]. In line with the development of extremely sensitive PCR-based techniques for a variety of pathogens of man and animals [18–21], an RT-nPCR for the detection of SVDV RNA has recently been described which is considerably more sensitive than virus isolation in tissue culture [22]. The advent of these new techniques, and the fact that the duration of infection with recent European strains of SVDV has not been investigated, prompted this study.

The aims of this investigation were to determine for how long SVDV and/or viral genome could be identified in the secretions, excretions and tissues of pigs following exposure to infection with the recent European isolate of SVDV, ITL/9/93.

MATERIALS AND METHODS

Viruses

SVDV strain ITL/9/93, isolated in 1993 from an outbreak of clinical disease in Forli, Italy, was used for the inoculation of pigs. The original isolate was passaged three times on IB-RS-2 cells (swine kidney cell line) [23] to achieve a titre suitable for experimental inoculation. After the final passage, the culture supernatant was clarified by centrifugation at 1000 g for 10 min, mixed with an equal volume of glycerol 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

Twenty-seven, 6-week-old, Landrace-cross pigs of approximately 20 kg in weight were used in this study. They were divided into 3 groups: 2 experimental groups, each of 12 pigs (Group 1: pigs 16–27; Group 2: pigs 28–39), and a negative control group of 3 pigs. The three groups were housed in separate pens in an isolation unit at the Institute for Animal Health, Pirbright Laboratory.

Experimental protocol

On the first day of the experiment (day 0) 2 out of the 12 pigs in each experimental group were inoculated with $10^{7.4}$ $TCID_{50}$ of SVDV/ITL/9/93 in 1 ml of tissue culture fluid by intradermal injection into the bulb of the heel of the right fore leg ('infected' pigs). The 10 remaining pigs in each experimental group were kept in contact with the directly-inoculated animals for the duration of the experiment ('exposed' pigs). For the first 7 days after inoculation, faeces were allowed to build up in the pens and there was minimal cleaning to ensure a high level of exposure to SVDV in the 'exposed' animals. Pigs were examined for clinical signs on dpi 3, 5, 7, 12, 14 and 21 and their rectal temperatures were measured daily. An objective assessment of the severity of clinical signs was made using the lesion scoring system of Mann [13] in which a pig showing severe lesions at all predilection sites is given a score of 100. The final lesion score for each animal was taken as the maximal score observed at any stage after infection. One animal from each group was killed at weekly intervals for the first 49 dpi then at monthly intervals from 63 dpi until all the remaining pigs were killed at 176 dpi. At 119 dpi, when SVDV and vRNA had not been detected in nasal swabs, faeces or tissues for 56 days, the remaining three pigs in each group were subjected to stress by being moved into a new, clean pen to form a single group of six animals.

Collection and processing of samples

Blood, nasal swabs and faeces were collected periodically throughout the experiment. Blood was collected

by venipuncture of the right anterior vena cava and allowed to clot. Serum was decanted and stored at −20 °C. Nasal swabs were collected by swabbing the nasal turbinates via the nostril using ENT swabs (Technical Service Consultant Ltd), which were then cut and placed in sterile bijou bottles containing 2 ml of phosphate-buffered saline (PBS) with 0·15% bovine serum albumin, 2 μg/ml Fungizone, 20 units/ml penicillin, 100 units/ml neomycin and 100 units/ml polymixin. Faeces were collected directly from the rectum. On each occasion that pigs were killed, the range of tissues listed in Table 1 was collected *post-mortem*. Samples of nasal swabs, faeces and tissues were snap-frozen on solid carbon dioxide and stored at −20 °C until processed.

Nasal swabs were thawed and ground with sterile sand in a pestle and mortar. The suspensions were centrifuged at 1000 g for 5 min. Between 1 and 20 g of faecal material was diluted 1:20 in PBS (w/v) and centrifuged at 5000 g at 4 °C for 20 min to remove particulate matter. The upper layer was decanted and centrifuged at 151200 g at 4 °C for 3 h to pellet the virus. The pellet was resuspended in 3 ml of PBS. A 20% (w/v) suspension of tissues was prepared in PBS by mincing with sterile scissors and grinding with sand in a pestle and mortar. The resulting suspension was centrifuged at 1000 g for 5 min. Final suspensions of nasal swabs and tissues, and the resuspended pellet obtained from faeces, were held on ice prior to vRNA extraction and virus isolation.

Virus isolation from nasal swabs, faeces and tissues

Suspensions of nasal swabs, faeces and tissues were treated with 1,1,2-trichloro-trifluoro-ethane (Freon; Sigma) as described by Sutmoller and Cottral [24] in order to maximize the likelihood of the recovery of virus. Freon-treated samples were inoculated onto five tubes of IB-RS-2 cell monolayers (0·2 ml/tube). After adsorption and rinsing with PBS, 2 ml of serum-free Eagle's medium (Glasgow) was added. The tubes were incubated at 37 °C for 3 days with rolling. Tubes were inspected daily for evidence of cytopathic effect (cpe). If cpe was observed, the supernatant was harvested, frozen and thawed once, and examined for the presence of SVDV by ELISA [25]. If there was no evidence of cpe after 3 days, the cell cultures were frozen and thawed once and a further two blind passages onto fresh tubes of IB-RS-2 cells were performed. The tubes were incubated for a further 5 days for each passage, inspecting the tubes daily as before. If no cpe was evident after the third passage, the sample was recorded as 'no virus detected'.

Measurement of antibody to SVDV

Sera were examined for total antibody to SVDV by the virus neutralization test (VNT) using as antigen SVDV/ITL/9/93 in the microneutralization assay described by Golding and colleagues [26]. Titres were defined as the reciprocal of the $\log_{10}$ dilution of the 50% end point using a virus dose of 100 $TCID_{50}$. Titres were classified according to the criteria defined by the Office International des Epizooties [27]) as positive ($\geqslant 1{\cdot}61$), doubtful ($\geqslant 1{\cdot}10$ but $< 1{\cdot}61$) or negative ($< 1{\cdot}10$). Isotype-specific antibodies (IgG and IgM) were measured by an adaptation of the isotype-specific indirect ELISA of Brocchi and colleagues [28] using monoclonal antibodies k139 3C8 for IgG (subtypes 1 and 2) and k52 1C3 for IgM (Mabs provided by Dr C. Stokes, University of Bristol).

The detection of SVD vRNA in nasal swabs, faeces and tissues

Total RNA was extracted from suspensions of nasal swabs, faeces and tissues and SVD vRNA was detected by RT-nPCR, as described by Lin and colleagues [22]. 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 genome.

Genomic and antigenic variation in sequential isolates

Total RNA was extracted from faeces and tissue culture fluid as described by Lin and colleagues [22]. Direct sequencing of the PCR-amplified fragments was performed using an f-mol® sequencing kit (Promega, UK). The primer oligonucleotides used for sequencing corresponded to regions of the SVDV genome coding for parts of the structural, capsid proteins VP3 and VP1 [22]. Selected virus isolates collected early or late in the course of infection were antigenically characterized in an indirect antigen-trapping ELISA (Samuel and colleagues) using Mab 5B7 [29], which recognizes antigenic site 2 [30], and Mab C29 [30] which binds to residue 261 of VP1, close to the carboxyl terminus of the protein.

Table 1. *Detection in tissues of SVDV by virus isolation and viral RNA by RT-nPCR*

Day killed pi	7		14		21		28		35		42		49		63		89		126*		176*			
Pig number	28	25	16	34	29	21	17	30	37	26	20	32	39	19	18	38	35	24	33	27	22	23	31	36
Tonsil	+†	+†	+‡	+†	+‡	−‡	+‡	+‡	+‡	−	−‡	−‡	+‡	−‡	−‡	+‡	−‡	−‡	−‡	−‡	−	−	−	−
Peyers' patches (ileal)	+‡	+‡	nd	+‡	−	−	−‡	−	−	−	−‡	−‡	−‡	−‡	−‡	−‡	−‡	−‡	−‡	−‡	−	−	−	−
Mesenteric LN	−‡	+†	nd	+‡	−	−	−‡	−	−	−	−‡	−‡	−	−	−‡	−‡	−‡	−	−‡	−‡	−	−	−	−
Prescapular LN	−+	+†	nd	+‡	−	−	−‡	−	−	−	−	−	−	−	−	−	−	−	−	−	nd	nd	nd	nd
Spleen	−‡	+†	nd	−‡	−	−	−	−‡	−	−	−	−	−	−	−	−	−	−	−	−	nd	nd	nd	nd
Large intestine (colon)	+‡	+†	nd	+‡	−	−	−	−	−	−	−	−	−	−	−‡	−‡	−‡	−	−	−	−	−	−	−
Small intestine (jejunum)	+‡	+‡	nd	+‡	−	−	−	−	−	−	−	−	−	−	−‡	−	−	−	−	−	−	−	−	−
Liver	−‡	+†	nd	+‡	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	nd	nd	nd	nd
Kidney	−‡	+‡	nd	nd	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	nd	nd	nd	nd
Myocardium	−‡	−‡	nd	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Somatic muscle	+†	+†	−‡	−‡	+‡	+‡	+‡	+‡	−‡	+‡	−‡	−‡	−	−‡	−	−	−	−	−‡	−‡	−	−	−	−
Brain stem	−‡	−‡	nd	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	nd	nd	nd	nd
Olfactory lobes	−‡	−‡	nd	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	nd	nd	nd	nd

Detection of SVDV and/or vRNA from a range of tissues collected *post mortem* from pigs killed at intervals following exposure to infection with SVDV/ITL/9/93. At 119 dpi the remaining pigs were subjected to stress by mixing the 2 initial groups to form a single group of 6 pigs in a new pen. Virus isolation was performed on all samples positive by RT-nPCR and on the additional samples shown on the table. *, after mixing; †, positive by virus isolation; ‡, negative by virus isolation; RT-nPCR: +, positive, −, negative; pi, post infection; nd, not done; LN, lymph node.

RESULTS

Clinical observations

All four 'infected' pigs which were inoculated intradermally developed clinical disease. Only 10 out of the 20 'exposed' pigs showed clinical signs (Table 2). Lesions were first observed in the directly-inoculated pigs between 4 and 7 dpi and in the exposed animals between 7 and 14 dpi. Maximal lesion scores were recorded on 7 dpi for the inoculated pigs and between 7 and 14 dpi for the exposed pigs. The severity of the clinical signs varied considerably between animals but was generally mild to moderate. There was no significant difference between the lesions scores of the two experimental groups (Mann Whitney U Test, $P > 0{\cdot}05$); the highest score in those pigs showing clinical signs was 66 and the lowest 1 (Table 2). Vesicle formation was associated with only mild lameness. Pyrexia was not observed in any pig (data not shown). Recovery was rapid and all pigs had returned to normal by 21 dpi.

Serology

Thirteen out of the 14 pigs which showed clinical signs were positive by VNT (Table 2, Fig. 1). The exception was pig 25 which was killed only 1 week after exposure to the 'infected' pigs before seroconversion could occur. Nine out the 10 animals which did not show clinical signs seroconverted by VNT (Table 2); the exceptional animal (pig 20) remained seronegative despite the fact that virus was isolated from its faeces intermittently until it was killed at 42 dpi (Table 3). The titres of subclinically infected animals were generally lower than those of pigs which showed clinical disease and for 3 subclinically infected animals (pig 21, 26 and 34) the maximum titre recorded was only classified as doubtful. The mean maximum titre of 1·9 $\log_{10}$ (S.D. 0·8) for the subclinically infected pigs was significantly lower than the mean maximal titre of 2·5 $\log_{10}$ (S.D. 0·7) for the clinically infected pigs ($t = 2{\cdot}07$, $P < 0{\cdot}05$, 22 D.F.). The antibody titres of the six pigs which were kept for at least 126 days are shown in Figure 1 and demonstrate the full range of responses observed. In pigs which showed a strong humoral response, titres rose rapidly within the first 28 dpi and remained positive at high titre until the animals were slaughtered up to 6 months after infection (e.g. pigs 22 and 33, Fig. 1). Titres in animals which showed only weak

Table 2. *Outcome of infection of pigs following exposure to a recent SVDV isolate ITL/9/93*

Day killed pi	7		14		21		28		35		42		49		63		89		126*		176*			
Pig number	28	25	16	34	29	21	17	30	37	26	20	32	39	19	18	38	35	24	33	27	22	23	31	36
Infection	I	E	I	E	I	E	I	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E
Clinical signs	+	+	+	–	+	–	+	–	+	–	–	+	+	–	+	+	+	–	+	–	+	–	+	–
Maximum lesion score	24	5	32	0	15	0	27	0	36	0	0	4	66	0	37	51	52	0	31	0	8	0	1	0
Seroconversion	+	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+
Maximum titre	1·3	1·0	2·8	1·3	2·1	1·3	2·7	1·9	2·5	1·2	0·7	2·8	2·2	1·9	2·8	2·7	2·7	1·8	3·4	3·0	3·1	2·7	2·8	2·7
FACS (dpi)	3	5	3	—	5	—	3	—	7	—	—	14	7	—	5	12	5	—	7	—	12	—	12	—

Clinical outcome (+, clinical signs; –, no clinical signs) in 24 pigs infected with SVDV/ITL/9/93 by either intradermal inoculation (infected pigs, I) or by exposure to the infected pigs (exposed pigs, E). The severity of clinical disease was assessed using the lesion scoring system of Mann (1982) in which the maximum score is 100. Sera were considered positive for antibody to SVDV if the reciprocal titre by VNT was equal to or greater than 1·61 $\log_{10}$ (Golding and colleagues, 1976). The maximum titre recorded at any stage following infection is shown. FACS, first appearance of clinical signs (dpi).

* After mixing.

pi, post infection.

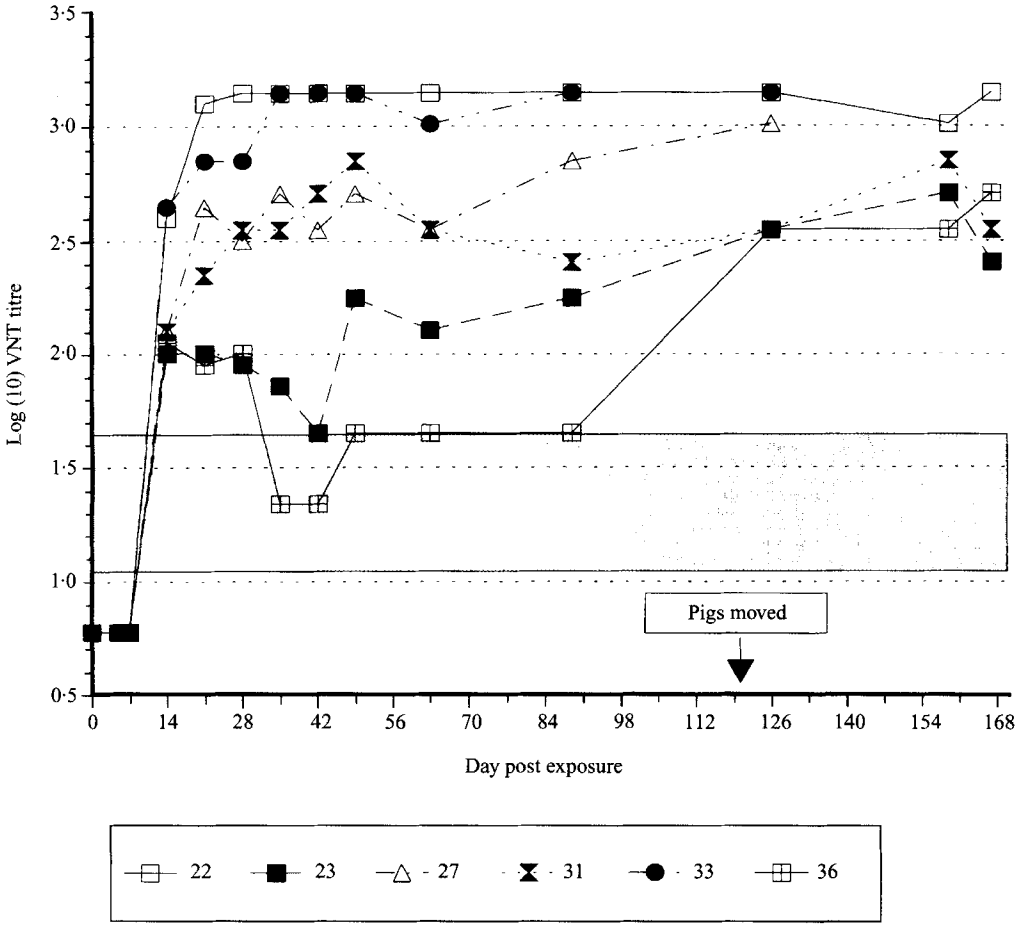

Fig. 1. SVDV-specific antibody was detected by VNT in pigs exposed to infection with SVDV/ITL/9/93 which showed either clinical (pig 22, 31, 33 or subclinical (pig 23, 27, 36) disease. Titres above the shaded area are classified as positive and those below, negative. Titres which fall within the shaded area are considered 'doubtful'.

serological responses also rose rapidly within the first 28 days, but subsequently fluctuated in the low positive or doubtful regions (e.g. pigs 23 and 36; Fig. 1).

After mixing of the remaining three animals in each group at 119 dpi to form a single group, an increase in antibody titre was observed in the two pigs (23 and 36) which had low antibody titres at the time of mixing. The other remaining pigs still had high antibody titres at this time and no further increases were observed.

Isotype-specific responses were studied for several pigs (Fig. 2). As expected, the early class of antibody to be detected was IgM which could be measured from about 7 dpi to 35 dpi. IgG was detected from about 14 dpi and was generally the predominant isotype detected from 21 days after the onset of seroconversion. Class switching from an early IgM response to a later IgG response occurred in pigs with both clinical (e.g. pig 22, Fig. 2) and subclinical (e.g. pig 27, Fig. 2) disease. An exception was pig 23 which was subclinically infected and only seroconverted to low titre by VNT following initial infection. Examination of sera by isotype-specific ELISA showed that the initial response was exclusively of IgM class and that the secondary response following mixing at 119 dpi was exclusively of IgG class (Fig. 2). Between the day 28 and day 126, pig 23 remained positive by VNT but negative by isotype-specifici ELISA, reflecting either a lower sensitivity for the ELISA or the detection of different antibodies in the two tests employed.

Identification of the agent

Samples collected in vivo

SVDV was detected in faeces by virus isolation and/or RT-nPCR in all the pigs examined at 7 dpi,

Table 3. *Detection of SVDV and viral RNA in nasal swabs, faeces and tonsils collected from pigs on the day of slaughter*

Day killed pi	7		14		21		28		35		42		49		63		89		126*		176*			
Pig number	28	25	16	34	29	21	17	30	37	26	20	32	39	19	18	38	35	24	33	27	22	23	31	36
RT-nPCR																								
Tonsil	††	††	‡‡	††	††	‡‡	††	††	††	–	‡‡	‡‡	††	‡‡	‡‡	††	‡‡	‡‡	‡‡	‡‡	–	–	–	–
Faeces	††	††	††	††	††	††	††	††	††	‡‡	††	††	‡‡	‡‡	††	‡‡	‡‡	‡‡	‡‡	††	‡‡	‡‡	‡‡	‡‡
Nasal swab	††	††	††	††	††	‡‡	††	††	–	‡‡	‡‡	–	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡

Detection of SVDV and/or vRNA in nasal swabs, faeces and tonsils collected from pigs on the day they were slaughtered. Animals were killed at the intervals shown following exposure to infection with SVDV/ITL/9/93 on day 0. at 119 dpi the remaining pigs were subjected to stress by mixing the 2 initial groups to form a single group of 6 pigs in a new pen.

* After mixing.

† Positive by virus isolation.

‡ Negative by virus isolation.

pi, post infection.

and in the majority at 14 dpi (Table 4). SVDV was detected in nasal swabs either by one or the other of the two techniques in the majority of samples collected at 7 and 14 dpi (Table 4). The number of samples which were positive by either technique declined with time. By virus isolation, SVDV was isolated intermittently from faeces for up to 63 dpi and from nasal swabs for up to 35 dpi. By RT-nPCR, vRNA was detected in both faeces and nasal swabs from at least one animal on every sampling occasion up to 63 dpi (Table 4). After 63 dpi and before mixing of the two groups at 119 dpi, neither virus nor vRNA could be detected in nasal swabs or faeces from the remaining pigs. As expected, mixing of the two groups resulted in fighting and, presumably, physiological stress. An apparent re-activation of virus excretion was detected as SVDV was again isolated from the faeces of 4 out of the 6 remaining animals at 121 dpi and faecal samples from all 6 were positive by RT-nPCR. Virus excretion was short-lived as SVDV could no longer be isolated from faeces collected 7 days or more after mixing, although two faecal samples collected at 126 dpi were positive by RT-nPCR alone. No SVDV was detected by either technique in nasal swabs collected at any time after mixing.

Samples collected post mortem

Swine vesicular disease virus was detected by virus isolation and/or RT-nPCR in the majority of tissues collected from pigs killed at 7 and 14 dpi (Table 1). Beyond 14 dpi the agent could not be detected by either technique in any of the tissues examined with the exception of tonsil and somatic muscle. Viral RNA, but not infectious virus, was detected in samples of tonsil and somatic muscle from some, but not all, of the pigs killed up to 63 and 35 dpi, respectively. SVDV was not detected by virus isolation or RT-nPCR in any of the tissues collected from pigs killed after 63 dpi (Table 1) and all samples collected from the three control animals remained negative for SVDV and vRNA throughout the experiment (data not shown).

The detection of SVDV and vRNA in nasal swabs, faeces and tonsils collected from pigs on the day of slaughter is collated in Table 3. In all cases where SVDV was identified in nasal swabs, the agent was also identified in the tonsils of the same pigs. However, the converse was not true and in three pigs (37, 38 and 39) vRNA was identified in tonsils *post mortem* but not in nasal swabs. There is, therefore, no correlation

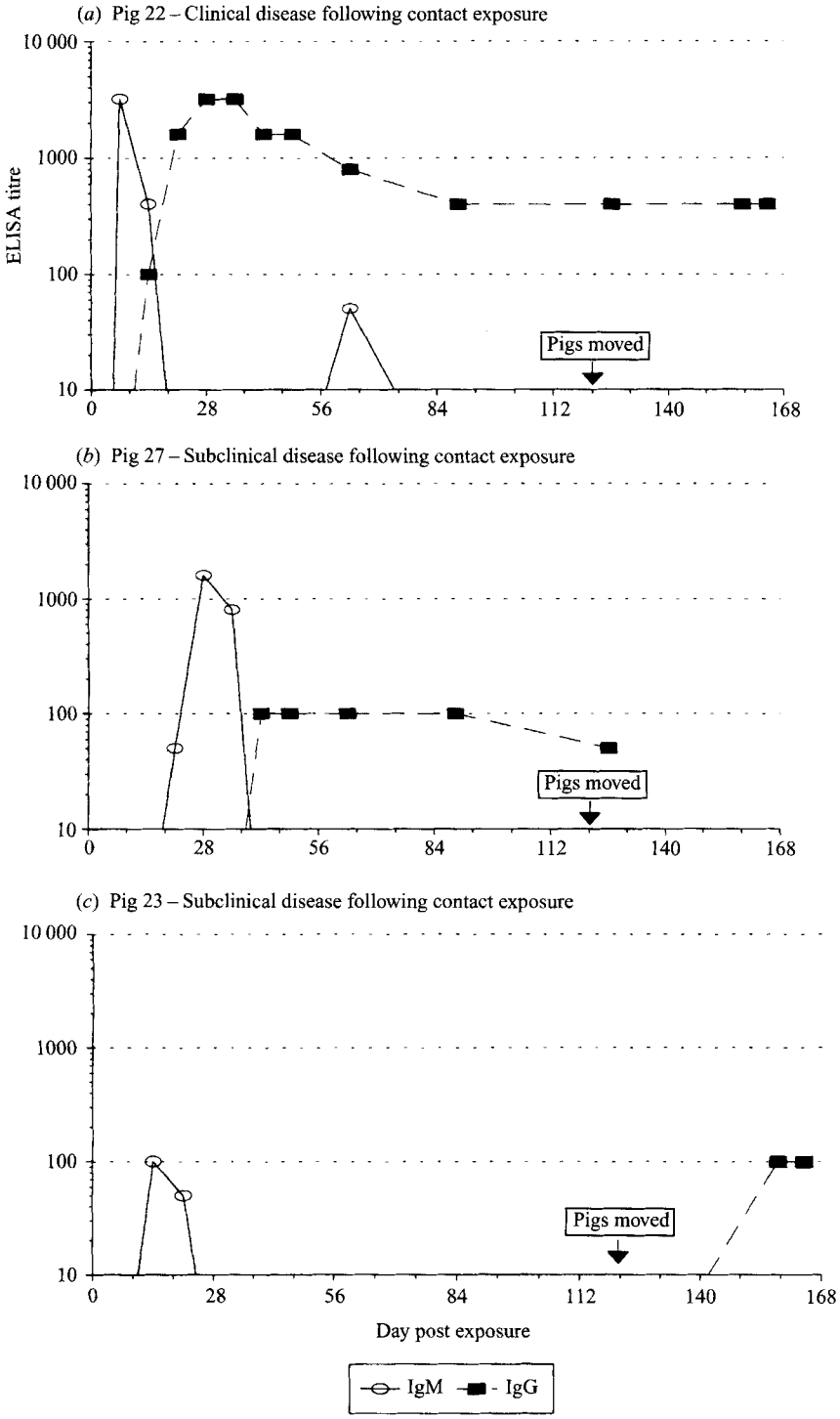

Fig. 2. SVDV-specific antibody as measured by isotype-specific ELISA in representative pigs showing clinical (Panel *a*: pig 22) or subclinical (Panel *b*: pig 27) disease. Pig 23 (Panel *c*) showed an unusual pattern with an early response of exclusively IgM class followed, after mixing of the remaining animals at 119 dpi, with a late response of exclusively IgG class.

between the continued presence of SVDV in the tonsil and its detection in nasal swabs. In four pigs (18, 21, 27 and 32) SVD vRNA was identified in the faeces at slaughter but the agent was not identified in the tonsils *post mortem*. In 2 out of these 4 animals SVDV was also isolated in tissue culture. Conversely, vRNA was detected in the tonsil but not in the faeces of pig 39. The tonsil does not, therefore, appear to be the only site of persistence for SVDV in animals in which the virus can be identified for prolonged periods.

Table 4. *Detection of SVDV by virus isolation and viral RNA by RT-nPCR in faeces and nasal swabs*

dpi	7	14	21	28	35	42	49	63	76	89	121*	126*	139*	159*	162*	167*
Faeces																
VI	17/17	11/12	7/12	4/15	2/13	2/12	0/8	1/7	0/8	0/8	4/6	0/6	0/4	ns	0/4	0/4
RT-nPCR	21/21	11/13	13/17	10/17	6/16	2/14	1/11	2/10	0/8	0/8	6/6	2/6	0/4	ns	0/4	0/4
Nasal swab																
VI	7/16	0/2	0/4	0/3	1/3	0/4	0/7	0/4	ns	0/3	ns	0/6	ns	0/4	ns	ns
RT-nPCR	16/24	17/22	4/20	3/18	2/16	3/14	5/12	2/10	ns	0/8	ns	0/6	ns	0/4	ns	ns

Detection of SVDV by virus isolation (VI) or SVD vRNA by RT-nPCR from faeces and nasal swabs collected from pigs exposed to infection with SVDV/ITL/9/93 on day 0. AT 119 dpi the remaining pigs were subjected to stress by mixing the 2 initial groups to form a single group of 6 pigs in a new pen. dpi, day post infection; ns, no sample; number of positive samples/number of samples examined.

* After mixing.

Genomic and antigenic variation in sequential isolates

The nucleotide sequence of part of the 1D gene of the virus was determined for isolates recovered from faeces collected during the course of the experiment (Fig. 3). Sequences were derived by direct sequencing of RT-nPCR amplicons. When the consensus sequence of the parental virus (SVDV, ITL/9/93/P, nomenclature detailed in legend to Fig. 3) was compared with each consecutive isolate without passage in tissue culture, the extent of variation ranged from 0% (18/F/7, 23/F/7, 27/F/7, 36/F/7 and 36/F/14) to 1·3% (18/F/63). Sequence changes were not detected in samples collected at 7 dpi. A maximum of 5 nucleotide substitutions were detected in an isolate recovered from faeces collected at 63 dpi (18/F/63). None of these nucleotide changes was conserved in viruses isolated at 121 dpi, but up to three base changes were detected at other sites. None of the five nucleotide changes which occurred in isolates recovered at 63 dpi resulted in amino acid substitutions (Fig. 4). In contrast, all three nucleotide changes detected in isolate 36/F/121 recovered at 121 dpi resulted in amino acid substitutions and two other isolates collected at 121 dpi also had the same substitutions at two out of these three sites (23/F/121 and 27/F/121). The extent of variation in amino acid sequence therefore varied from 0% (18/F/7, 18/F/14, 18/F/63, 23/F/7, 23/F/21, 27/F/7, 27/F/21, 27/F/28, 27/F/63, 36/F/7 and 36/F/14) to 1·9% (36/F/121). There was no change in plaque morphology in tissue culture between isolates which were recovered from faeces collected at 63 dpi and 121 dpi (data not shown).

Cross-neutralization assays (Table 5) showed that sera collected from pigs 31 and 36 early in the course of infection (7 dpi) neutralized the parental virus, and virus isolates from the same animal at early or late stages of infection, equally well. Likewise sera collected from either pig late in the course of infection (167 dpi) also had equally high neutralizing titres against the parental virus and viruses isolated from the homologous pig at 7 or 121 dpi. Early and late isolates from pigs 31 and 36 were also examined using Mabs (Table 5). Mab C29 bound early isolates from either pig at least as well as the parental virus but did not bind to the late isolates from either pig. Conversely, Mab 5B7 bound the parental virus, early isolates and late isolates equally well.

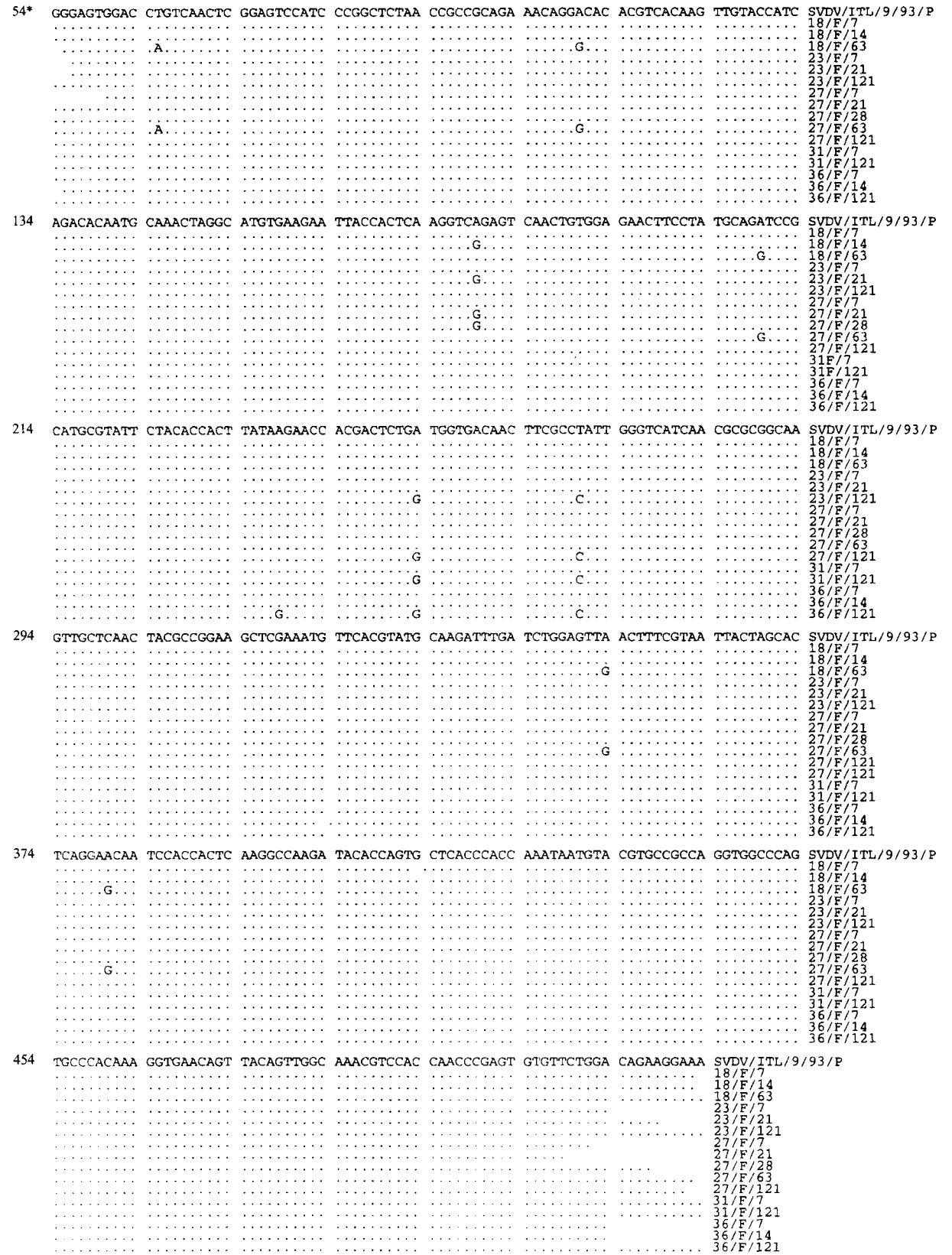

Fig. 3. Nucleotide sequences encoding the VP1 region of the parental strain ITL/9/93 and selected isolates from experimental pigs collected at increasing time after infection. Differences in sequence between the parental virus and isolates derived from the experimental animals are shown. The nomenclature used for viruses was as follows: Pig number/origin/dpi; F, faeces; P, parental virus. *, Numbering according to SVDV/UKG/27/72 [31].

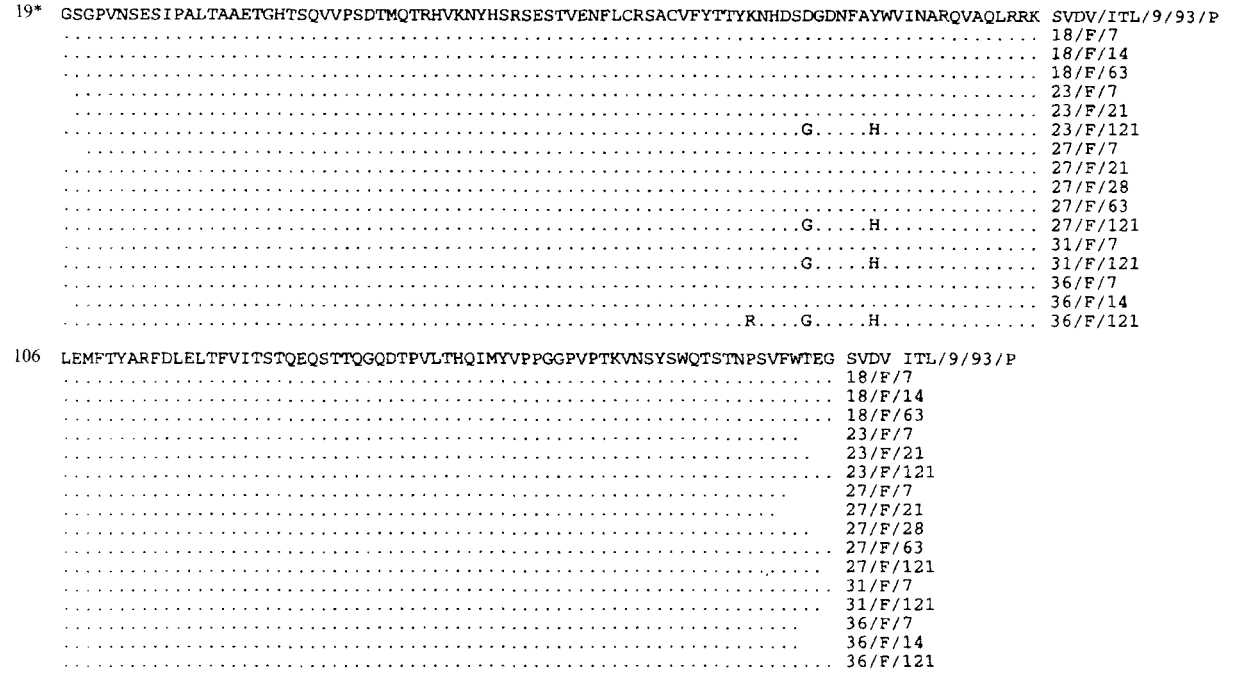

Fig. 4. Comparison of the amino acid sequences of the VP1 domain deduced from the corresponding nucleotide sequences shown in Fig. 3. Only amino acids that differ from those of the parental virus are indicated. *, Numbering according to SVDV/UKG/27/72 [31].

Table 5. *Antigenic comparison between parental virus (SVDV/ITL/9/97/P) and isolates from early and late in the course of infection*

	VNT titres of sera collected at		Percent reactivity in ELISA compared with parental virus	
Virus	21 dpi	167 dpi	C29	5B7
SVDV/ITL/9/93/P	2·3	2·5	100	100
31/F/7/T	2·5	2·3	> 100	> 100
31/F/121/T	2·1	2·5	18	> 100
SVDV/ITL/9/93/P	1·9	2·7	100	100
36/F/7/T	1·8	2·1	> 100	> 100
36/F/121/T	1·8	2·3	10	> 100

VNT titres of sera collected from pigs 31 and 36 at 21 and 167 dpi against the parental virus (SVDV/ITL9/93) and against isolates collected from the same pigs either 7 (31/F/7/T, 36/F/7/T) or 121 (31/F/121/T, 36/F/121/T) dpi. The nomenclature used for viruses was as follow: Pig number/Origin/dpi/tissue culture isolate; F, faeces; P, parental virus; T, virus isolate passaged in tissue culture. The same viruses were also examined in an antigen trapping ELISA using Mabs C29 and 5B7 as detecting antisera. As defined by Samuel [29], ELISA reactivity was classified as very high (> 76%), high (46–75%) or none (< 20%).

DISCUSSION

Swine vesicular disease virus was recovered in the nasal secretions and faeces of pigs exposed to infection with a recent European strain of SVDV, ITL/9/93, for up to 63 dpi. This contrasts with the majority of previous reports where SVDV was detected in faeces up to a maximum of 39 dpi [12] and confirms the work of Gourreau and colleagues [14] demonstrating that SVDV can be present in the excretions and secretions of pigs for several months after infection. Using conventional techniques for virus isolation from tissues *post mortem*, SVDV has previously been detected for up to 14 dpi [14, 32]. Virus was isolated

from tissues for the same length of time in the study reported here. However, using RT-nPCR, vRNA was detected for up to 63 dpi in tonsils and for up to 35 dpi in somatic muscle. The ability to detect SVDV for longer by RT-nPCR than by virus isolation was due either to the greater sensitivity of the RT-nPCR [22] or because vRNA persists for longer in tissues than viable virus. The stringent precautions which were taken to eliminate the possibility of false-positive results by RT-nPCR have been detailed elsewhere [22] making this an unlikely explanation for RT-nPCR positive/virus isolation negative results. In only one (Table 3; pig 20, faeces) of the many RT-nPCR negative samples that were examined by virus isolation was virus recovered from a sample which was negative by RT-nPCR. It therefore appears that there were rare instances in which substances inhibitory to Taq DNA polymerase were still present after RNA extraction from the faeces. As not all RT-nPCR negative samples were examined by virus isolation, it is possible that the results under-represent slightly the extent of virus excretion in faeces.

Virus excretion in faeces was 'reactivated' for a short period in pigs from which SVDV could no longer be identified by subjecting the animals to the physiological stress of mixing. Re-excretion was short-lived as the agent could not be identified beyond one week after mixing. SVDV must, therefore, have persisted in one or several of the remaining pigs between 63 and 119 dpi without being detected in faeces or nasal swabs collected *in vivo* or in tissues collected *post mortem*. The finding that virus was recovered beyond 28 dpi suggests the carrier state may occur in SVD. The possibility that reactivation of virus excretion resulted from uptake of virus persisting in the environment can be discounted due to the absence of the agent from any samples collected for a period of over 60 days and due to the fact that the animals were moved to a new, clean pen at the time of mixing. The demonstration of a rise in antibody titre in 2 of the 6 pigs following mixing is further evidence of a reactivation of infection resulting in immune stimulation.

Infectious virus and vRNA were detected for considerably longer in faeces than in tissues and nasal swabs. This finding is in agreement with previous reports for both SVDV [14, 15] and human enteroviruses [33] and points towards a site of virus persistence associated with the alimentary tract. Of the tissues examined, SVDV and vRNA were identified for longest in the tonsil. However, the tonsil was not the only site of persistence as virus was detected in tissue culture from the faeces but not the tonsils of four pigs on the day they were killed (Table 3) and virus was not detected from the tonsils of pigs slaughtered at the time of reactivation of excretion. Sites of virus persistence other than the tonsil were not identified.

Clinically, the disease produced by SVDV/ITL/9/93 was mild. The morbidity observed in this study was lower than in many previous reports of similar studies [9, 10, 13]. Differences in morbidity may be related to the use of different strains, methods of administration, amounts of virus and the environmental conditions under which the animals were kept. Rough flooring and wet conditions underfoot can influence the severity of lesions [12]. Fifty per cent of the animals exposed to infection with SVDV/ITL/9/93 by contact showed only a subclinical infection. This is consistent with subclinical disease being a common sequel to infection of both man and animals with enteroviruses [1, 2, 4, 13, 33, 34]. Infection with SVDV by the oral route, as presumably occurred here, has been shown preferentially to result in subclinical, rather than clinical disease [12]. Antibody titres in subclinically infected pigs were significantly lower than in clinically affected pigs. In one animal (pig 20) seroconversion did not occur but virus was recovered in the faeces for up to 42 dpi (Table 3, additional data not shown). SVDV is very resistant to environmental factors such as pH and temperature, and can survive transit through the stomach. Prolonged isolation of virus could, therefore, be due to transfer of virus from other affected pigs. However, it is more likely that the virus replicated at some site in the gastro-intestinal tract without producing significant pathology or inducing a strong immune response. There are reports that human enteroviruses can persist in affected tissues (myocardium) without causing gross cytopathology or consistently eliciting a humoral or cellular immune response [35, 36].

The epidemiological significance of subclinical infection in SVD is unclear. There is only 1 report of the transmission of infection from subclinically infected pigs to susceptible animals placed in contact [12] and there are no reports of clinical disease arising from such sources of virus. In the study reported here virus was recovered for prolonged periods from both clinically and subclinically affected pigs suggesting that the establishment of persistence was not related to the clinical course of infection. The fact that virus was shed into the environment suggests that there is at

least the theoretical possibility of the transmission of infection from subclinically infected pigs or from persistently infected animals which have recovered from clinical disease. Experiments to demonstrate or refute the possibility of transmission are currently underway at the Institute for Animal Health, Pirbright. If it is shown that transmission of infection or disease can occur this will have profound implications for eradication campaigns. Control currently relies on the detection of SVD due to the appearance of clinical signs or due to findings seropositive animals during serological surveillance. In the experiment reported here, virus was recovered from four pigs which did not show clinical disease and in which the maximum antibody titres by VNT were so low as to be classified as doubtful (pig 21, 26 and 34) or negative (pig 20). If such animals arise in the field they might well be missed using current surveillance procedures and would therefore represent an untraceable source of infection.

For the establishment of the carrier state, antigenic variation may be one of the mechanisms by which persistent viruses evade the immune response of the host. Cross neutralization studies demonstrated that SVD viruses collected early or late in the course of infection were neutralized equally well by serum collected at the start of the humoral response or shortly before the pigs were killed (Table 5). Therefore, the virus used in the current experiment did not alter during the course of infection in such a way that it was no longer neutralized effectively. All 3 externally exposed capsid proteins of SVDV contain regions which are antigenic [37]. Isolates collected late in the course of infection revealed up to a maximum of 3 amino acid substitutions in VP1, suggesting that subtle alterations in antigenicity did occur. These substitutions were mapped onto the three-dimensional structure of coxsackievirus B3 [8, 38]. Of these substitutions, two (positions 80 and 85) were located in, or close to, the BC loop region of VP1 which constitutes antigenic site 1 in SVDV [37]. The third substitution (position 91) is predicted to lie in β-sheet C and is located close to position 261 which has been found to be critical for the binding of neutralizing monoclonal antibody C29 [30]. The finding that Mab C29 showed no binding with viruses having substitutions at position 91 suggests that alterations in or near Site 1 might be linked in some way to an ability to persist in the host. The demonstration that a Mab to Site 2 (5B7) bound early and late isolates equally well suggests that this site is not involved in persistence.

In conclusion, the current study demonstrated that a recent isolate of SVDV and vRNA could be recovered from the tonsils of pigs, and from their secretions and excretions, for much longer than has been accepted for historic strains of the virus. Further experiments are underway to determine the frequency with which the carrier state occurs and to identify the sites in which the virus persists. If the carrier state is shown to be a common sequel to infection with SVDV, this will significantly affect our understanding of the epidemiology of the disease.

ACKNOWLEDGEMENTS

This work was funded by the United Kingdom Ministry of Agriculture, Fisheries and Food. We would like to acknowledge G. Hutchings and G. Wilsden for technical help and L. Fitzpatrick and B. Taylor for their skilled husbandry of the animals. Our thanks go to Dr A. Donaldson for guidance and critical review of the manuscript and to Dr K. Sestak for his initial work in this area. We are grateful to Dr E. Brocchi of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy for supply of Mab 5B7.

REFERENCES

1. Nardelli L, Lodetti E, Gualandi GL, et al. A foot-and-mouth disease syndrome in pigs caused by an enterovirus. Nature, London 1968; **219**: 1275–6.
2. Mowat GN, Darbyshire JH, Huntley JF. Differentiation of a vesicular disease of pigs in Hong Kong from foot-and-mouth disease. Vet Rec 1972; **90**: 618–21.
3. Dawe PS, Forman AJ, Smale CJ. A preliminary investigation of the swine vesicular disease epidemic in Britain. Nature, London 1973; **241**: 540–2.
4. Knowles NJ, Sellers RF. Swine vesicular disease. In: Beran GW, ed. Handbook of zoonoses, Section B: Viral, 2nd edn, Boca Raton: CRC Press Inc. 1994: 437–44.
5. FAO Animal Health Yearbooks 1992–1996, Food and Agriculture Organization of the United Nations, Rome, 1992–1996.
6. Brocchi E, Zhang G, Knowles NJ, et al. Molecular epidemiology of recent outbreaks of swine vesicular disease: two genetically and antigenically distinct variants in Europe, 1987–1994. Epidemiol Infect 1997; **118**: 51–61.
7. Zhang G, Wilsden G, Knowles NJ, McCauley JW. Complete nucleotide sequence of a coxsackie B5 virus and its relationship to swine vesicular disease virus. J Gen Virol 1993; **74**: 845–53.
8. Knowles NJ, McCauley JW. Coxsackievirus B5 and the relationship to swine vesicular disease virus. In:

Tracy S, Chapman NM, Mahy BWJ, eds. The coxsackie B viruses: current topics in microbiology and immunology. Berlin: Springer-Verlag, 1997; **223**: 153–67.
9. Dekker A, Moonen P, de Boer-Luijtze EA, Terpstra C. Pathogenesis of swine vesicular disease after exposure of pigs to an infected environment. Vet Microbiol 1995; **45**: 234–50.
10. Lai SS, McKercher PD, Moore DM, Gillespie JH. Pathogenesis of SVD in pigs. Am J Vet Res 1979; **48**: 463–8.
11. Burrows R, Mann JA, Goodridge D. Swine vesicular disease: comparative studies of viruses isolated from different countries. J Hyg 1974; **73**: 109–17.
12. Mann JA, Burrows R, Goodridge D. Mild and subclinical infections with swine vesicular disease virus. Bull Off int Epizoot 1975; **83**: 117–22.
13. Mann JA. The pathogenesis of swine vesicular disease [Thesis]. University of Reading, 1982.
14. Gourreau JM, Berthand N, Mishra V, Jaco B, Vallet C. Persistence of swine vesicular disease virus in the pig. Recueil Med Vet (Alfort) 1975; **151**: 283–7.
15. Johnston RS. SVD: an appraisal of the eradication policy. State Vet J 1977; **32**: 1–17.
16. Loxam JG, Hedger RS. Swine vesicular disease: Clinical signs, diagnosis, epidemiology and control. Rev Sci Tech Off Int Epiz 1983; **2**: 11–24.
17. Anon. Proceedings of the Second Annual Meeting of EU National Reference Laboratories for SVD, Brussels, Belgium, 19–20 February, 1996.
18. Zoll GJ, Melchers WJG, Kopecka H, Jambroes G, Vanderpoel HJA, Galama JMD. General primer-mediated polymerase chain reaction for detection of enteroviruses: application for diagnostic routine and persistent infections. J Clin Microbiol 1992; **38**: 160–5.
19. Severini GM, Mestroni L, Falashi A, Camerini F, Giacca M. Nested polymerase chain reaction for high-sensitivity detection of enteroviral RNA in biological samples. J Clin Microbiol 1993; **13**: 1345–49.
20. Donn A, Martin LA, Donaldson AI. Improved detection of persistent foot-and-mouth disease infection in cattle by the polymerase chain reaction. J Virol Meth 1994; **49**: 179–86.
21. Baxi MK, Borchers K, Bartel T, Schellenbach A, Baxi S, Feield HJ. Molecular studies of the acute infection, latency and reaction of equine herpesvirus-1 (EHV-1) in the mouse model. Virus Res 1996; **40**: 33–45.
22. Lin F, Mackay DKJ, Knowles NJ. Detection of swine vesicular disease virus by reverse transcription-polymerase chain reaction. J Virol Meth 1997; **65**: 111–21.
23. De Castro MP. Behaviour of the foot-and-mouth disease virus in cell cultures: susceptibility of the IB-RS-2 line. Arch Inst Biol São Paulo 1964; **31**: 63–78.
24. Sutmoller P, Cottral GE. Improved techniques for the detection of foot-and-mouth disease virus in carrier cattle. Arch ges Virusforsch 1967; **21**: 170–7.
25. Ferris NP, Dawson, M. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular disease. Vet Microbiol 1988; **16**: 201–9.
26. Golding SM, Hedger RS, Talbot P, Watson J. Radial immuno-diffusion and serum-neutralization techniques for assay of antibodies to SVD. Res Vet Science 1976; **28**: 142–7.
27. OIE manual of standards for diagnostic tests and vaccines, 2nd edn. Paris, France: Office International des Epizooties, 1992; 21–7.
28. Brocchi E, Berlinzani A, Gamba D, De Simone F. Development of two novel monoclonal antibody-based ELISAs for the detection of antibodies and the identification of swine isotypes against swine vesicular disease virus. J Virol Meth 1995; **52**: 155–67.
29. Samuel AR, Knowles NJ, Samuel GD, Crowther JR. Evaluation of a trapping ELISA for the differentiation of foot-and-mouth disease virus strains using monoclonal antibodies. Biologicals 1991; **19**: 299–310.
30. Nijhar SK. Antigenic characterisation of swine vesicular disease virus [thesis]. University of Hertfordshire, 1997.
31. Seechurn P, Knowles NJ, McCauley JW. The complete nucleotide sequence of a pathogenic swine vesicular disease virus. Virus Res 1990; **16**: 255–74.
32. Burrows R, Mann JA, Goodridge D, Wrathall AE, Done JT. SVD-study in pregnant sows. Zentrablatt fur Veterinarmedizin B 1977; **24**: 177–82.
33. Rotbart HA, Romero JR. Laboratory diagnosis of enteroviral infections. In: Rotbart HA, ed. Human Enterovirus infections. Washington, DC: American Society for Microbiology, 1995: 401–18.
34. Burrows R, Greig A, Goodridge D. Swine vesicular disease. Res Vet Science 1973; **15**: 141–4.
35. Archard LC, Bowles NE, Cunningham L, et al. Molecular probes for detection of persisting enterovirus infection of human heart and their prognostic value. European Heart J 1991; **12** Suppl D: 56–9.
36. Bowles NE, Rose ML, Taylor P, et al. End-stage dilated cardiomyopathy: persistence of enterovirus RNA in myocardium at cardiac transplantation and lack of immune response. Circulation 1989; **80**: 1128–36.
37. Kanno T, Inoue T, Wang Y, Sarai A, Yamaguchi S. Identification of the location of antigenic sites of swine vesicular disease virus with neutralization-resistant mutants. J Gen Virol 1995; **76**: 3099–106.
38. Muckelbauer JK, Kremer M, Minor I, et al. The structure of coxsackievirus B3 at 3.5 Angstrom resolution. Structure 1995: **3**: 653–67.