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Molecular epidemiology of recent outbreaks of swine vesicular disease: two genetically and antigenically distinct variants in Europe, 1987–94

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SUMMARY

Viruses from the recent epidemic of swine vesicular disease (SVD) in Europe have been isolated and characterized by antigenic and genetic methods to examine the likely epidemiological origins of the disease. Antigenic analysis was performed on 77 SVD viruses (SVDV) isolated in Europe between 1966 and 1994 using two panels of monoclonal antibodies (MAb) in a trapping ELISA. Genetic analysis of 33 of the SVD viruses by reverse transcription-polymerase chain-reaction (RT-PCR) amplification and nucleotide sequencing of the 1D (VP1) coding region was also performed. Comparison of the nucleotide sequences with each other and with three other previously published SVDV sequences revealed four distinct groups which correlated exactly with the results of the pattern of reactivity with MAbs. The first group consisted solely of the earliest SVD virus isolated (ITL/1/66) while the second group comprised viruses present in Europe and Japan between 1972 and 1981. The third group consisted of viruses isolated from outbreaks of SVD in Italy between December 1988 and June 1992. Viruses isolated between 1987 and 1994 from Romania, the Netherlands, Italy and Spain formed a fourth group. The genetic and antigenic similarity of the most recent virus isolates from Western Europe to a virus isolated in Romania 5 years previously suggests that the possible origin of the recent epidemic of swine vesicular disease in Western Europe was in Eastern Europe.

INTRODUCTION

Swine vesicular disease (SVD) is a highly contagious disease of pigs which is caused by a virus belonging to the genus *Enterovirus* within the family *Picornaviridae*. Swine vesicular disease virus (SVDV) is antigenically closely related to the human pathogen coxsackievirus B5 (CB5) [1, 2]. Recently the complete genome sequences of three SVD viruses and a CB5 virus have been described [3–6] and the relationship between SVDV and CB5 virus was confirmed but considerable divergence of nucleotide sequence was seen throughout the virus genome.

Swine vesicular disease was first recorded in

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October 1966 when a vesicular disease appeared simultaneously on two farms in Lombardy, Italy which had received pigs for fattening from a common origin. The outbreak was diagnosed as foot-and-mouth disease (FMD) on clinical grounds but the failure to confirm this diagnosis by laboratory tests led to a study that showed the causative agent to be an enterovirus [7]. No further cases of the disease occurred in 1966.

Swine vesicular disease was next encountered in Hong Kong in 1971 [8] and retrospective studies revealed the presence of the virus as early as May 1970 (N. J. Knowles, unpublished observations). Outbreaks in Hong Kong have continued almost annually until 1991 [9].

In October and December 1972 a number of outbreaks of SVD occurred in Italy: in Naples, Rome, Latina, Matera and Ravenna between September and December [10]. During December 1972 SVD was also confirmed in Austria [11], the United Kingdom [12], France [13] and Poland [11]. In 1973 the first outbreaks were recorded in West Germany [14], Switzerland [15], Belgium [10] and Japan [16]. Between 1975 and 1983 outbreaks of SVD appeared sporadically in France (1975, 1982–83), the Netherlands (1975) [17], Malta (1975) [18], Japan (1975) [19], Belgium (1979) [21]. In Germany and UK occasional outbreaks occurred almost annually until 1982 despite the imposition of strict hygiene and control measures. In Italy SVDV appeared to persist and small numbers of outbreaks occurred nearly every year until 1992.

In early July 1992, SVD was reported in breeding stock on two neighbouring farms in Ede, Netherlands and more than 1000 pigs were destroyed [22]. Later in the month SVD was detected on a third farm in the same area. In October 1992, the disease was found on three additional farms in the Netherlands, two of which were close to those involved in the July outbreaks. The sixth farm to be affected was located in Putten and the virus was thought to have been introduced by a vehicle which had been associated with two of the outbreaks at Ede. Nearly 2000 pigs were destroyed in the latter three outbreaks. No new outbreaks occurred until January and February 1994 when SVD reappeared in the Netherlands at a pig collecting centre at Haarle, Overijssel.

In June 1992 five outbreaks of SVD occurred in Italy. In August 1992 two outbreaks were recorded in the Modena and Siena provinces: both involving animals belonged to the same consignment of pigs imported from the Netherlands. It was apparent (*vide infra*) that these were antigenically distinct from previously encountered viruses.

In October 1992, during a SVD serological survey of pigs imported into Belgium from the Netherlands, sera containing antibodies to SVDV were found in fattening pigs originating from a farm in West Flanders Province, Belgium. In early February 1993 SVDV antibodies were detected in pigs on a farm in Antwerp Province, Belgium. Again the infection was thought to have been introduced by the purchase of piglets from the Netherlands. In both cases no clinical disease was seen and no virus could be isolated.

An epidemiological survey of SVD in Spain was undertaken and began in November 1992. In all, 94 farms in 39 different towns had imported pigs from the Netherlands and one farm, in Lerida Province, had pigs with antibodies to SVDV.

In February 1993, an outbreak of a vesicular condition was reported on a farm in the Lerida Province, Spain, and the entire herd was slaughtered. In April 1993, SVD was diagnosed at two other farms in Huesca Province in north-eastern Spain and the pigs were slaughtered (a total of 3278 head). No outbreaks of SVD had occurred in Spain since April 1993 and more than 10000 pigs have since been tested for SVDV antibodies [23, 24].

It had been suspected that SVD outbreaks occurred during the 1970s in some Eastern European countries. The first outbreaks recorded in Austria in 1972 were associated with the importation of pigs from Poland where the virus was also isolated [11]. We have learned of a number of outbreaks of SVD in the former Eastern Bloc following improved communication with these countries and virus from one outbreak in Romania in 1987 has been analysed in this study.

These events are summarized in Table 1 which shows the number of SVD outbreaks which have occurred in Europe since 1987.

De Simone and colleagues [25], using two panels of monoclonal antibodies, showed that it was possible to adduce that SVDV fell into four antigenic groups: group 1 consisted of the virus from the first outbreak (ITL/1/66); group 2 consisted of strains which circulated in Europe in the early 1970s; group 3 was composed of Italian strains from 1988 until December 1992, and group 4 was composed of viruses which had been present in Europe since July 1992. This paper reports the detailed antigenic and genetic analysis of viruses isolated between 1987 and 1994 from epidemics of SVD in Europe and a comparison with earlier strains. Four congruent groupings were found

Table 1. *Swine vesicular disease outbreaks in Europe, 1987–94**

	Year							
Country	1987	1988	1989	1990	1991	1992	1993	1994
Romania	⩾1†							
Italy		1	1		6	31	11	30
Netherlands						6		3
Belgium						1‡	1‡	
Spain							3	

* Data was derived from OIE Disease Information reports, except:
† M. Danes, personal communication, 1994.
‡ Confirmed by serological testing, no virus isolated.

in both the antigenic and genetic properties of the virus.

MATERIALS AND METHODS

Viruses

The SVD virus isolates studied are listed in Table 2.

Monoclonal antibody analysis (IZSLE, Brescia)

Two panels of MAbs prepared against SVDV strains R178 (isolated in Italy in 1973) and R1046 (ITL/2/91) (Brocchi and colleagues, unpublished observations) were used in a trapping ELISA as previously described [26]. Essentially each MAb was reacted with virus grown in IB-RS-2 cells which had been trapped using a pre-titrated polyclonal rabbit SVDV antiserum. The results were expressed as a percentage of the homologous reaction for each MAb after first standardizing against a polyclonal guinea-pig SVDV antibody. Multivariate analysis was performed using the Unistat™ Statistical Package version 1·2 (Unistat Ltd., London, UK). This consisted of a hierarchical cluster analysis using the average between groups. Distance was measured up by the squared Euclid method where:

$$\text{Distance}\,(x, y) = \sum_i (x_i - y_i)^2.$$

Nucleotide sequence determination (IAH, Pirbright)

Viruses examined in this study (ITL/1/66, ITL/2/73, ITL/A/89, ITL/1-2/91, ITL/1/92, ITL/7-10/93, ITL/12/93, ITL/1-8/94, NET/1/75, NET/1-3/92, NET/1-4/94, ROM/1/87 and SPA/1/93; Table 2) were grown in tissue culture flasks containing approximately 5×10^7 IB-RS-2 cells. Total RNA was extracted from infected cells and first strand cDNA synthesis was carried out as described previously [6] using the oligonucleotide GSVD-1 (Table 3) in a final reaction volume of 20 μl. The product was extracted with phenol, precipitated with ethanol and resuspended in 15 μl of water. A 5 μl sample was amplified by PCR with Taq polymerase (Boehringer-Mannheim, Germany), 200 nM of the oligonucleotides (GSVD-3 and NK44, Table 3), 200 μM deoxynucleotides and buffer as supplied by the manufacturer, in a reaction volume of 50 μl. Cycling conditions were: 95 °C, 4 min; 50 °C, 90 s; 72 °C, 90 s; followed by 25 cycles of 94 °C, 60 s; 50 °C, 60 s; and 72 °C, 60 s. An aliquot of the product was analysed by electrophoresis through an agarose gel. The remainder was purified by adsorption to and elution from a silica matrix (Magic PCR preps™, Promega, WI). Sequencing was done essentially as described in the fmol™ sequencing kit (Promega, WI) using approximately 100 mg of template DNA and oligonucleotides labelled at the 5′ terminus with ^{32}P-γATP (Table 3).

Nucleotide sequence determination (BFAV, Tübingen)

Each virus examined (D6494/73, D6533/75, D6629/81, R1004, R1071 (ITL/2/92), NL 92; Table 2) was passaged once on porcine secondary embryonic kidney (PSEK) cells and then used to infect a 25 cm² flask of cells. Total RNA was extracted when CPE was observed, as described previously [27, 28]. Following hybridization of 20 μg of infected cell RNA with 50 ng of primer 3-R (Table 3), first strand cDNA synthesis was carried out with 10U of avian myeloblastoma virus reverse transcriptase (Stratagene, CA). Amplification was carried out with primers 1-F and 1-R (Table 3). Taq polymerase (Promega, WI), in a Bio-Med Thermocycler 60 (Bachofer, Germany). Thirty cycles of 1 min at 93 °C, 2 min at 55 °C and 5 min at 72 °C were performed. The PCR amplification products were analysed by agarose gel electrophoresis. The products were then treated with Klenow enzyme and T4 polynucleotide kinase, purified with Geneclean II (Bio-101, La Jolla, CA) and concentrated fivefold. Sequencing was performed using a commercially available sequencing kit (USB, CA). PCR amplification products exhibiting significant numbers of

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Table 2. *Designation and origin of the swine vesicular disease viruses studied by MAb analysis and nucleotide sequencing*

Virus designation					
WRL ref. no.*	Other number†	Geographic origin	Date collected	Genetic group	MAb group
		West Germany			
None	D6494/73	Krefeld, Northrhine-Westphalia	September 73	II	nd
None	D6533/75	Bersenbrück, Lower-Saxony	August 75	II	nd
None	D6629/81	Freudenstadt, Baden-Württemberg	September 81	II	nd
		Italy			
ITL/1/66	Italy/66	Lombardy	October 66	I	1
ITL/2/73	None	Rome, Lazio	December 72	II	nd
None	R178	Padova, Veneto	06/04/73	nd	2
ITL/A/89	R967	Campodoro, Padova, Veneto	23/12/88	III	3a
None	R1004	Portici, Naples, Campania	26/05/89	III	3b
ITL/1/91	R1042	Messina, Sicily	30/01/91	III	3c
ITL/2/91	R1046	Agerola, Naples, Campania	15/04/91	III	3c
None	R1053	Alfonsine, Ravenna, Emilia Romagna	09/06/92	nd	3c
None	R1056	Ischie, Naples, Campania	16/06/92	nd	3c
None	R1057	Riolo Terme, Ravenna, Emilia Romagna	17/06/92	nd	3c
ITL/1/92	R1058	Campogalliano, Modena, Emilia Romagna	27/06/92	III	3c
None	R1061	Castevetro, Modena, Emilia Romagna	13/08/92	nd	4c
None	R1062	Montepulciano, Siena, Tuscany	14/08/92	nd	4c
None	R1063	Montepulciano, Siena, Tuscany	14/08/92	nd	4c
None	R1064	Spilamberto, Modena, Emilia Romagna	14/08/92	nd	4c
None	R1065	Spilamberto, Modena, Emilia Romagna	14/08/92	nd	4c
None	R1068	Chiamicelle, Siena, Tuscany	24/08/92	nd	4c
None	R1069	Poggio D., Siena, Tuscany	24/08/92	nd	4c
None	R1070	Castiglione del Lago, Perugia, Umbria	28/08/92	nd	4c
ITL/2/92	R1071	Fano, Pesaro, Marche	02/09/92	IV	4c
None	R1072	Ripat., Chieti, Abruzzo	11/09/92	nd	4c
None	R1073	Castiglione del Lago, Perugia, Umbria	17.09/92	nd	4c
None	R1076	Pozzilli, Isernia, Molise	20/09/92	nd	4c
None	R1079	S. Giovanni Incarico, Frosinone, Lazio	10/10/92	nd	3e
None	R1080	Centallo, Cuneo, Piemonte	01/10/92	nd	4c
None	R1083	Erchie, Brindisi, Puglia	22/10/92	nd	3d
None	R1086	Luzzara, Reggio Emilia, Emilia Romagna	05/11/92	nd	4c
None	R1087	Cusano Mutri, Benevento, Campania	16/11/92	nd	4c
None	R1092	Serradifalco, Caltanissetta, Sicily	07/12/92	nd	3d
None	R1093	Condofuri, Reggio di Calabria, Calabria	07/12/92	nd	4c
None	R1094	Luzzara, Reggio Emilia, Emilia Romagna	11/12/92	nd	4c
None	284/93	Nola Naples, Campania	01/02/93	nd	4c
ITL/7/93	534/93	Nola, Naples, Campania	22/02/93	IV	nd
ITL/8/93	R1098	Potenza, Basilicata	04/03/93	IV	4c
None	R1105	Tursi, Matera, Basilicata	12/03/93	nd	4c
ITL/9/93	R1120	Bertinoro, Forli, Emilia Romagna	18/03/93	IV	4c
None	R1150	Matera, Basilicata	March 93	nd	4c
ITL/10/93	R1159	Barano d'Ischia, Naples, Campania	10/04/93	IV	4c
ITL/12/93	None	Ariccia, Rome, Lazio	May 93	IV	4c
ITL/13/93	R1164	Ariccia, Rome, Lazio	01/06/93	nd	4c
None	R1168	Cosenza, Calabria	23/06/93	nd	4c
None	R1172	Davoli, Cosenza, Calabria	18/11/93	nd	4c
None	R1173	Mescia R., Foggia, Puglia	28/12/93	nd	4c
ITL/1/94	R1174	S. Felice Rubiera, Reggio Emilia, Emilia Romagna	24/01/94	IV	4c
None	R1175	Albano L, Potenza, Basilicata	02/02/94	nd	4c
None	R1176	Potenza, Basilicata	02/02/94	nd	4c
None	R1177	Campomaggiore, Potenza, Basilicata	02/02/94	nd	4c

Table 2—(*cont.*)

Virus designation					
WRL ref. no.*	Other number†	Geographic origin	Date collected	Genetic group	MAb group
None	R1178	Grottole, Matera, Basilicata	10/02/94	nd	4c
ITL/2/94	R1179	S. Giorgio Lucano, Matera, Basilicata	10/02/94	IV	4c
None	R1180	Castiglione del Lago, Perugia, Umbria	14/02/94	nd	4c
None	R1181	Pisticci, Matera, Basilicata	17/02/94	nd	4c
None	R1182	Pianopoli, Cosenza, Calabria	16/02/94	nd	4c
None	R1183	Nemoli, Potenza, Basilicata	26/02/94	nd	4c
None	R1184	Lattarico, Cosenza, Calabria	05/03/94	nd	4c
ITL/3/94	R1185	Bettona, Perugia, Umbria	05/03/94	IV	4c
None	R1186	S. Giorgio Lucano, Matera, Basilicata	08/03/94	nd	4c
ITL/4/94	R1187	Soncino, Cremona, Lombardy	13/04/94	IV	4c
None	R1188	Torre Pallavicina, Bergamo, Lombardy	13/04/94	nd	4c
ITL/5/94	R1189	Salsomaggiore, Parma, Emilia Romagna	29/04/94	IV	4c
None	R1190	Fidenza, Palma, Emilia Romagna	05/05/94	nd	4c
None	R1191	Tricarico, Matera, Basilicata	05/05/94	nd	4c
ITL/6/94	R1193	Mirandola, Modena, Emilia Romagna	11/05/94	IV	4c
ITL/7/94	R1194	Amantea, Cosenza, Calabria	15/05/94	IV	4c
ITL/8/94	R1195	Macomer, Nuore, Sardinia	18/05/94	IV	4c
None	R1196	Bardi, Palma, Emilia Romagna	24/05/94	nd	4c
None	R1197	Poggio Rusco, Mantova, Lombardy	25/05/94	nd	4c
None	R1198	Pavullo, Modena, Emilia Romagna	03/06/94	nd	4c
None	R1200	Agro di Bella, Potenza, Basilicata	29/09/94	nd	4c
None	R1201	Cavriana, Mantova, Lombardy	22/11/94	nd	4d
None	R1202	Guidizzolo, Mantova, Lombardy	07/12/94	nd	4d
None	R1203	Guidizzolo, Mantova, Lombardy	07/12/94	nd	4d
None	R1204	Bozzolo, Mantova, Lombardy	08/12/94	nd	4d
		Japan			
None	J1′73‡	Ibaraki	November 73	II	nd
None	H3′76‡	Hokkiado	February 76	II	nd
		Netherlands			
NET/1/75		Uden, North Brabant Province	17/11/75	II	2
NET/1/92	1992-1	Ede, Gelderland	03/07/92	IV	4b
NET/2/92	1992-2	Ede, Gelderland	03/07/92	IV	4c
NET/3/92	1992-3	Ede, Gelderland	04/08/92	IV	4c
None	NL 92	Ede, Gelderland	1992	IV	4c
NET/1/94	1994-1	Haarle, Overijssel	28/01/94	IV	4c
NET/2/94	1994-2	Haarle, Overijssel	February 94	IV	4c
NET/3/94	1994-3		March 94	IV	4c
NET/4/94§		Rosmalen	February 94	IV	nd
		Romania			
ROM/1/73			1973	II	2
ROM/1/87			1987	IV	4a
		Spain			
SPA/1/93		Vallfogona de Balaguer, Lerida	February 93	IV	4c
		United Kingdom			
UKG/27/72‡		Staffordshire	11/12/72	II	2

* World Reference Laboratory for Foot-and-Mouth Disease reference number.
† Reference number used in the country of origin.
‡ Sequences previously published [3–5].
All viruses were isolated in their country of origin except § isolated in WRL, Pirbright, UK.

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Table 3. *Designation, sequence and location of the oligonucleotide primers used for PCR amplification and sequencing of the SVDV isolates studied*

Oligonucleotide name	Sequence (5′ → 3′)	Position on the SVDV genome*		Used for
IAH, Pirbright				
GSVD-1	GTCTGCTGGGGGTGTTGAT	3D	5979–5960	RT of RNA
GSVD-3	ACACCCTTTATAAAACAGG	1C	2414–2432	PCR + sequencing
NK44	CCACACACAGTTTTGCCAGTC	2A	3394–3374	PCR-sequencing
NK45	GCCAACGTACACGGCACC	2A	3334–3317	Sequencing
GSVD-5	AACATGCTGTATGCGTTGCCTAT	1D	3027–3005	Sequencing
GSVD-6	GTCAAACCTGGCCCACCCGTCATA	1D	3052–3029	Sequencing
BFAV, Tübingen				
3-R	TCAAATGTGACTGGATAGTGCTT	2A	3525–3503	RT of RNA
1-F	CGACAACTTCGCCTACTGGGT	1D	2704–2724	PCR-sequencing
1-R	CTTCCCACACACAGTTTTGCCAGTC	2A	3398–3374	PCR + sequencing

* Numbering according to the sequence of SVDV/UKG/27/72 [4].

ambiguous bases were cloned in *E. coli* using the pSPT18 vector as previously described [29].

Phylogenetic analysis

Nucleotide sequences were analysed on an IBM compatible personal computer using programmes written by one of the authors (NJK). All pairwise comparisons were performed by giving each base substitution equal statistical weight (ambiguities were ignored). A binary tree was constructed according to sequence relatedness across the interval of nucleotides 315 to 504 of the 1D (VP1) gene (genomic bases 2762 to 2951) using the NEIGHBOR-JOINING method as implemented in the computer program NEIGHBOR and a dendrogram plotted using the program DRAWGRAM both from the PHYLIP version 3.5c phylogeny package [30].

RESULTS

Antigenic analysis

Antigenic analysis was conducted on 81 SVD viruses from Europe. The analysis was performed using a trapping ELISA with 2 panels, each of 10 monoclonal antibodies, raised against 2 viruses from Italy (R178 and R1046 [ITL/2/91]). The panels contained neutralizing and non-neutralizing antibodies. A summary profile of reactivity with the panels of antibodies is shown in Figure 1. Four of the non-neutralizing antibodies (3F5, 2B1, 5B8 and 4E8) show variable reactions with tissue culture supernatants of some viruses which were dependent on the integrity of the virus particle. As previously indicated [25], four distinct patterns of antigenicity could be demonstrated. It was apparent that a new virus population had appeared in Italy in August 1992 which had a similar profile of reaction with monoclonal antibodies to the profile displayed by viruses from the Netherlands earlier in June of the same year. Hierarchical cluster analysis (average between groups) confirmed that there were four antigenic groups and these are shown in Figure 2. The clusters consisted of one group with only member (ITL/1/66), the second group consisted of viruses from the early 1970s, the third group contained viruses from Italy isolated between 1988–91, and the fourth group consisted of viruses isolated from Western Europe between July 1992 and the end of 1994 and a Romanian virus isolated in 1987.

Nucleotide sequence analysis by RT-PCR and direct sequencing

The nucleotide sequences of the SVDV isolates have been submitted to the EMBL database at the European Bioinformatics Institute, Hinxton, UK (World Wide Web address: http://www.ebi.ac.uk/). Complete 1D gene sequences were not obtained for all SVDV isolates which was probably due to the specificity of the internal sequencing primers. Additionally the nucleotide identity at some positions was unreadable, either due to premature polymerase termination or to the presence of more than one equal intensity band, possibly indicating a polymorphism. Notwithstanding these reservations, phylogenetic analysis of the nucleotide sequences of those viruses

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	Monoclonal antibodies raised against SVDV Italy/73											Monoclonal antibodies raised against SVDV Italy/71									Viruses
Neutralization	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−	−	+	+/−	−		
Group	4H9	5D9	3B8	5A10	4H3	1H1	5B7	3F5	2B1	5B8	2G7	1G1	6E1	2D2	3G8	2H11	4C5	1A2	2H8	4E8	
1	100	100	0	100	100	0	0	50	0	0	0	0	0	0	0	0	0	100	100	100	ITL/1/66
2	100	100	100	100	100	100	100	100	100	100	0>25	0	0	0	0	25	25>50	100	100	100	R178 (Italy 1973), UK/27/72, NET/1/75, ROM/1/73
3A	0	0	100	100	100	100	100	100	100	100	100	100	0	100	100	100	100	100	100	100	R967 (Italy 1988)
3B	0	0	100	50	50	100	100	100	100	100	100	100	0	100	100	100	100	100	100	100	R1004 (Italy 1989)
3C	0	0	50	0	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	ITL/1/91, ITL/2/91, ITL/1/92, R1053, R1056, R1057
3D	0	50	50	0	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	R1092 (Italy 1992)
3E	0	0	50	0	0	100	100	100	100	100	100	100	100	100	100	100	100	0	0	100	R1079, R1083 (Italy 1992)
4A	0	0	0	100	100	0	100	100	100*	0	0	0	0	0	0	0	0	100	100	100	ROM/1/87
4B	25	25	0	100	100	25	100	25	100*	100*	0	0	0	0	0	0	0	0	0	100*	NET/1/92
4C	0>50	0>50	0	100	100	0>50	100	100*	100*	100*	0	0	0	0	0	0	0	100	100	100*	Europe 1992-94 (59 isolates, see Table 2)
4D	25	25	0	25	100	25	100	100*	100*	100*	0	0	0	0	0	0	0	100	100	100*	R1201, R1202, R1203, R1204 (Italy 1994)

Fig. 1. Reactivity patterns of SVD viruses with two panels of anti-SVDV monoclonal antibodies. Reactivity of virus with antibody was measured by a capture ELISA as described by Samuel and colleagues [26]. Virus reactivity was grouped according to the percentage reactivity of antibody with the sample virus compared with the reactivity to the virus against which antibody was raised. An asterisk following 100% indicates that 100% reactivity was seen only following treatment of virus at 56 °C for 30 min. Values indicated $0 > 25$ and $0 > 50$ indicate that variable reactivities of some viruses within a sub-group between 0 and either 25% or 50% of the homologous reactivity were observed. The virus designation is shown in Table 2.

for which complete (or nearly complete) 1D sequences were obtained was undertaken and the results are shown in Figure 3*a*. Additionally a dendrogram was constructed for a shorter region of the 1D genes (nucleotides 315–504; genome sequence 2761–2950) for which a larger number of sequences were available (Fig. 3*b*). Although this tree suggests that viruses belonging to group II could be further subdivided, this is not supported by the analysis of either the longer sequences (Fig. 3*a*) or another study using a larger data set (G. Zhang, D. T. Haydon, N. J. Knowles, J. W. McCauley, unpublished observations). The trees were not corrected for multiple substitutions since their effects are minimal at this level of sequence diversity. In both trees there are four distinct branches (clades): one which consists of the first virus isolated in Italy in 1966 (group I), one of viruses from 1972–82 (group II), one of viruses from 1988–92 (group III) and one of viruses from Western Europe between 1992 to 1994 and the single virus from Romania isolated in 1987 (group IV). For some other viruses only partial sequences of the 1D coding region were obtained, however, these all fell into one of the four genetic groups (see Table 2). The results show that the viruses from Western Europe between 1988 and 1994 formed two quite separate groupings with a wide sequence divergence and it is considered very unlikely that a virus from group III was the progenitor of viruses of group IV.

Nucleotide sequence analysis following cloning

In some cases nucleotide sequences were determined by preparing cloned PCR products. Several plasmids containing inserts were obtained and sequenced. Two virus isolates were sequenced in this manner (D6629/81 and R1071 [ITL/2/92]) and some heterogeneity between clones of the same virus was detected. The sequence of D6629/81 consisted of two sequences (varying by 2·5%) which might be due to the error-prone polymerase activities of reverse transcriptase and the DNA polymerase from *Thermus aquaticus* used to produce the clones or alternatively because two closely related viruses were present in an outbreak. However, the analysis of R1071 (ITL/2/92) showed a wider diversity with two distinct SVDV sequences: one was encountered in two clones and was similar to the group III sequences and the other was found seven times and was related to the group IV sequences. The sequences were approximately 15% different and would be very unlikely to be due to the

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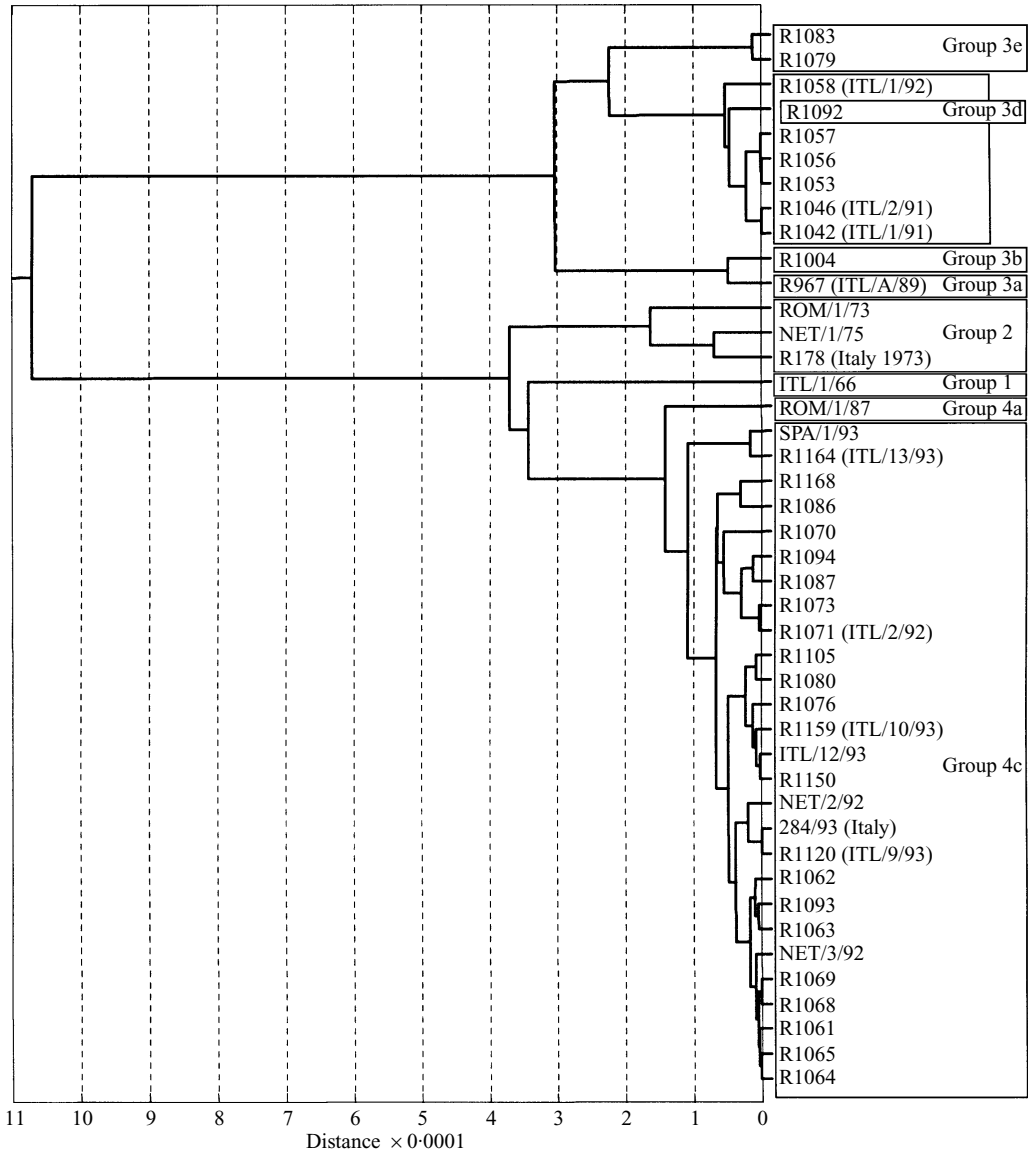

Fig. 2. Dendrogram of the antigenic relationships between the SVD viruses examined using a MAb ELISA. The dendrogram was prepared as described in the materials and methods. Virus designation is shown in Table 2.

use of error-prone polymerases. It is difficult to exclude the possibility of laboratory contamination but it is also feasible that the pig was infected with two different viruses or that the epithelial tissue from which the virus was isolated had become contaminated at the abattoir during collection. Monoclonal antibody profiles of uncloned virus however revealed only the presence of virus from antigenic group 4. In the case of D6629/81, in which one sequence determined from a cDNA clone was identical to that of a virus isolated in Germany in 1975 (D6533/75), we cannot rule out laboratory contamination. Due to the reservations we hold about these two viruses, they have not been included in the dendrograms shown in Figure 3 but their omission does not significantly influence the above conclusions.

DISCUSSION

From 1972–94 SVDV outbreaks were reported in Europe, and viruses isolated from many of these outbreaks have been subjected to analysis with monoclonal antibodies and by sequencing a region of the virus RNA genome. Two sequences of SVDV from Japan were also included in the genetic comparison.

A comparison of the SVDV nucleotide sequences in the region of the genome that encodes the virus capsid

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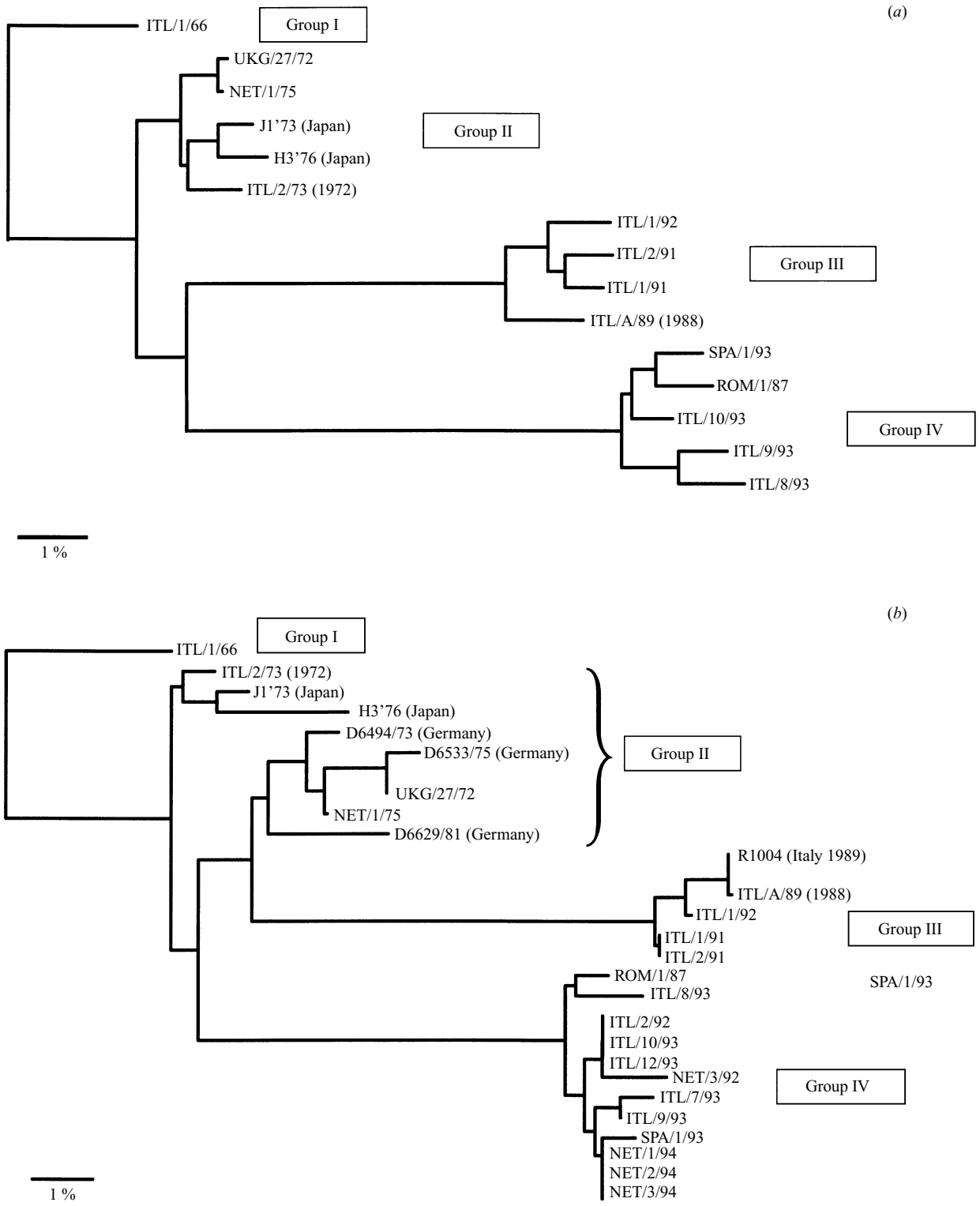

Fig. 3. Phylogenetic tree derived from VP1 RNA sequences from SVDV. The designation of the viruses is shown in Table 2. The tree reconstructions were done using PHYLIP version 3.5 [30] as described in the materials and methods using the program NEIGHBOR. ITL/1/66 was used as an outgroup to root the trees. (a) NEIGHBOR-JOINING tree of viruses for which the complete VP1 sequence was determined. (b) NEIGHBOR-JOINING tree of SVD viruses reconstructed from VP1 bases 315–504 (genome sequence 2761–2950). Where WRL reference numbers do not match the year of isolation, the year is included in parentheses.

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polypeptide 1D was made and a dendrogram of the relationships was constructed (Fig. 3). Similar dendrograms were made from the data from monoclonal antibody analysis (Fig. 2). The analysis of the European and Japanese SVD viruses showed that they fell into four distinct groups. The earliest SVD virus isolated, ITL/1/66, was distinct from all the other viruses examined, although there was less genetic distance to the viruses from the 1970s than to those isolated later. SVD viruses which predominated in Europe during the 1970s, when infection was widespread, were eliminated from most countries by rigorous control measures. During the late 1980s and 1990s two groups emerged: one present in Italy from 1988 to June 1992 (group III) and another in Netherlands, Italy and Spain from July 1992 to 1993 (group IV). It seems unlikely that viruses typical of group III (1988–92) were the first ancestors of those belonging to the later group (IV) because of their wide sequence divergence. The monoclonal antibody dendrogram has four groups, albeit group 3 can be easily divided into five subgroups and group 4 into four subgroups (Figs. 1 and 2). The ability to subdivide these two groups may be a reflection of the selection of the strain of virus against which the two panels of MAbs used were raised. Although the relationships between monoclonal antibody profiles can be used to group antigenic variants, it is less evident that they can be used to interpret population genetic and evolutionary trends. Nevertheless, our observation that virtually congruent virus groups resulted from using the two methods reinforces our conclusions that two populations of SVDV, with distinct origins, have recently been circulating in Europe.

Neither the virus that seeded the outbreaks nor the geographical location of the initial focus of infection can be firmly established. Epidemiological data suggest that the SVD viruses in group IV first appeared in Western Europe in the Netherlands. During 1993 seven consignments of pigs received for slaughter in Italy were found to be positive for SVD virus; all originated in the Netherlands [31]. However, it seems likely that the origin of viruses of group IV was Eastern Europe. A strain of SVDV belonging to this group was present in Romania in 1987. It seems unlikely that SVDV had remained undetected in Western Europe because recent serological surveys in all EU countries have only detected the presence of SVDV antibodies in Italy [32]. SVDV has been present in Eastern Europe: it was known that outbreaks of SVD occurred in Poland during 1972 and 1973 [11] and it has also been recently reported that outbreaks occurred in USSR in 1973 and 1975 (V. V. Drygin, personal communication, 1994), Bulgaria in 1971 (Y. Ivanov, personal communication, 1994) and Romania in 1973 (M. Danes, personal communication, 1994). Whether the virus lineage of genetic group IV evolved from SVDV previously introduced into Eastern European or is a recent introduction into that area from either Hong Kong or China has not been resolved.

In summary and conclusion: genetic and antigenic data has shown that two lineages of SVDV have been present in Western Europe since 1988. The nucleotide sequence diversity of the earlier one (group III) makes it unlikely that it gave rise to the later one. We submit that the origin for this later lineage (group IV) may be Eastern Europe and that it probably evolved subsequently in Italy.

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