



ELSEVIER

Veterinary Microbiology 73 (2000) 137–157

**veterinary  
microbiology**

www.elsevier.com/locate/vetmic

## Genetic typing of classical swine fever virus

D.J. Paton<sup>a,\*</sup>, A. McGoldrick<sup>a</sup>, I. Greiser-Wilke<sup>b</sup>,  
S. Parchariyanon<sup>c</sup>, J.-Y. Song<sup>d</sup>, P.P. Liou<sup>e</sup>, T. Stadejek<sup>f</sup>,  
J.P. Lowings<sup>a</sup>, H. Björklund<sup>g,1</sup>, S. Belák<sup>g</sup>

<sup>a</sup>*Veterinary Laboratories Agency – Weybridge, Addlestone, Surrey KT15 3NB, UK*

<sup>b</sup>*Institute of Virology, Veterinary School Hannover, Buenteweg 17, 30559 Hannover, Germany*

<sup>c</sup>*National Institute of Animal Health, Bangkok, Bangkok 109000, Thailand*

<sup>d</sup>*National Veterinary Research and Quarantine Service, 480 Anyang 6 Dong, Anyang 430-016, South Korea*

<sup>e</sup>*Taiwan Animal Health Research Institute, 376 Chung-Cheng Road, Tansui, Taipei 25101, Taiwan*

<sup>f</sup>*National Veterinary Research Institute, P-24-100 Pulawy, Poland*

<sup>g</sup>*Department of Virology, National Veterinary Institute, Biomedical Center,  
PO Box 585, S-751 23 Uppsala, Sweden*

### Abstract

Three regions of the classical swine fever virus (CSFV) genome that have been widely sequenced were compared with respect to their ability to discriminate between isolates and to segregate viruses into genetic groups. Sequence data-sets were assembled for 55 CSFVs comprising 150 nucleotides of the 5' non-translated region, 190 nucleotides of the E2 envelope glycoprotein gene and 409 nucleotides of the NS5B polymerase gene. Phylogenetic analysis of each data-set revealed similar groups and subgroups. For closely related viruses, the more variable or larger data-sets gave better discrimination, and the most reliable classification was obtained with sequence data from the NS5B region. No evidence was found for intertypic recombination between CSFVs. A larger data-set was also analysed comprising 190 nucleotides of E2 sequence from 100 CSFVs from different parts of the world, in order to assess the extent and global distribution of CSFV diversity. Additional groups of CSFV are evident from Asia and the nomenclature of Lowings et al. (1996) [Lowings, P., Ibata, G., Needham, J., Paton, D., 1996. *J. Gen. Virol.* 77, 1311–1321] needs to be updated to accommodate these. A tentative assignment, adapting rather than overturning the previous nomenclature divides CSF viruses into three groups with three or four subgroups: 1.1, 1.2, 1.3; 2.1, 2.2, 2.3; 3.1, 3.2, 3.3, 3.4. The expanding data-base of CSFV sequences should improve the prospects of disease tracing in the future, and provide a basis for a standardised approach to ensure

\* Corresponding author. Tel.: +44-1932-357285; fax: +44-1932-357239.

E-mail address: dpaton.wood.cvl@gtnet.gov.uk (D.J. Paton)

<sup>1</sup> Present address: Division of Genetics and Physiology, Department of Biology, University of Turku, FIN-20014 Turku, Finland.

that results from different laboratories are comparable. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Classical swine fever virus; Epidemiology; Pestivirus; Diversity; Phylogenetics

---

## 1. Introduction

Classical swine fever (CSF) is a highly contagious disease that is mainly spread by contacts between live pigs or by feeding pigs with contaminated pig meat. Both the domestic pig and wild boar can become infected. Controls are mostly based on vaccination or stamping out. Although eradicated from many countries, CSF continues to cause serious problems in different parts of the world (Edwards et al., 2000).

The causative agent, classical swine fever virus (CSFV) is a Pestivirus of the family Flaviviridae. It has an enveloped virion incorporating glycosylated membrane proteins and an icosahedral nucleocapsid. The genome is a single strand RNA of positive sense, approximately 12,300 nucleotides in length. It has a non-translated region at either end (5'NTR and 3'NTR), encompassing a single open reading frame encoding a large protein that is cleaved into smaller fragments. The genes encoding the structural proteins are found towards the 5' end of the genome, and include the major envelope glycoprotein gene E2. The genes encoding non-structural proteins are located mainly in the 3' two thirds of the genome, and include the polymerase gene NS5B (Meyers and Thiel, 1996). The complete nucleotide sequence is available for 14 CSF viruses, filed under the following Genbank accession numbers: J04358 (Alfort, Meyers et al., 1989); M31768 (Brescia, Moormann et al., 1990); D49532/3 (ALD/GPE<sup>-</sup>, Ishikawa et al., 1995); Z46258 (C-strain, Moormann et al., 1996); X87939 (Alfort-187, Ruggli et al., 1996); X96550 (CAP, Tratschin unpublished); U45477/8 (Riems/Glentorf, Muller unpublished); L49347 (P97, Liu unpublished); AF091507 (HCLV, Wang unpublished); AF091661 (Brescia, Kyle unpublished); AF092448 (Shimen, Huang unpublished); U90951 (Alfort A19, Smondack unpublished).

The Pestivirus genus comprises three genotypes in addition to CSFV, namely bovine viral diarrhoea virus types I and II (BVDV I and II) and ovine border disease virus (BDV). These pestiviruses occasionally infect pigs but usually do not spread efficiently outside their ruminant hosts. Despite close similarities to BVDV and BDV, CSF viruses form a distinctive group that can be differentiated serologically or on the basis of genetic similarities (Paton, 1995).

Technological advances have facilitated genetic typing of viruses based on the determination and comparison of nucleotide sequences for fragments of viral genomes. This approach can be used to establish the relatedness of different virus isolates which is useful for classification and can help trace patterns of virus spread, exposing weaknesses in control strategies. To be fully effective as a means of tracing the spread of CSF, genetic typing results must be interpreted in the light of the overall extent and geographic distribution of CSFV variability. A starting point for an assessment of the diversity of CSFV is a representative collection of the viruses that may be encountered in different parts of the world. At the same time that viruses are collected, it is essential that

information is gathered on the epidemiology of the outbreaks. This can provide hypotheses for the likely causes of virus persistence and spread, which can be evaluated by genetic typing. Furthermore, the genetic comparison of isolates obtained from a series of outbreaks with known linkages can be used to validate the interpretation of the genetic typing and to determine the rate of virus mutation in the field. The largest collection of CSFVs is held at The European Reference Laboratory for CSF at Hannover in Germany (Greiser-Wilke et al., 2000).

Genetic typing has proved very useful as a means of tracing the spread of CSFV and is generally considered superior to antigenic methods (Lowings et al., 1994, 1999; Fritzemeier et al., 1999; Stadejek et al., 1997; Hofmann and Bossy, 1998; Vilcek and Paton, 1998; Bartak and Greiser-Wilke, in press). The method has been used to demonstrate: (1) virus dissemination from a point source of introduction; (2) transmission between domestic pigs and wild boar; (3) transmission across national frontiers; (4) outbreaks of differing virulence associated with very closely related viruses; (5) local persistence of particular variants, most likely in infected wild boar; (6) differentiation between field and vaccine viruses. The studies have also demonstrated that the sequences of different regions of the CSFV genome are rather stable during virus transmission between outbreaks.

The discrimination possible between isolates depends on the length and variability of the target region of the genome that is used for comparisons. There is an ongoing debate concerning which regions of the genome should be used for genetic comparisons, and which methods of comparison are most meaningful. Although different regions may be suitable, a small number of standard approaches should be selected so that data sets from different laboratories are comparable. Studies on the phylogenetic relationship of CSFVs have divided the viruses into two main groups based on sequence comparisons of different genomic regions (Lowings et al., 1996; Vilcek et al., 1996, 1999; Björklund et al., 1999) with two additional disparate isolates — Kanagawa and Congenital Tremor. Different nomenclatures have been used to describe the various virus groups and subgroups, but the method of Lowings et al. (1996) is most widely used. According to this system, Group 1 comprises most of the historical isolates and Group 2 most of the current ones. Recently, additional distinct viruses have been reported from the Far East (Parchariyanon et al., 1998; Sakoda et al., 1999) and it is necessary to consider how these compare to existing groupings. This paper provides an update of progress on the development of genetic typing as an adjunct to CSF control. Aspects covered include the need to standardise on the choice of genetic targets, and on the methods of phylogenetic analysis, and current knowledge on the extent and distribution of CSFV diversity.

## 2. Methods

### 2.1. *Virus collections and sequence data*

Much sequence data for this study was obtained from viruses held at the European Community Reference Laboratory in Hannover (Greiser-Wilke et al., 2000) and much has already been published (Lowings et al., 1996; Vilcek et al., 1996; Greiser-Wilke et al.,

Table 1  
Origins of newly analysed viruses and viruses not recorded in Hannover database

Name of isolate	Place of isolation	Year of isolation	Provider/(Comment)
88015, 88039, 88136	Korea	1988	Jae-Young Song
97009, 97347	Korea	1997	Jae-Young Song
96939, 96940	Korea	1996	Jae-Young Song
LOM, E-ALDKOR	Korea	Historical	Jae-Young Song (vaccine strain)
Ukr1/94	Japan	Historical	Jae-Young Song
	Ukraine	1994	Stadejek, T. (from wild boar)
	(Ivano-Frankowsk)		
Ukr101/97	Ukraine (Izmailov)	1997	Stadejek, T. (from wild boar)
P97	Taiwan	1997	Liu, S.-T. (Genbank L49347)
93/4, 94/4	Taiwan	1993/1994	Liou, P.P.
Evi136, Evi100	Brazil	1987	Roehe, P.
Thai viruses	Thailand	Historic	Parchariyanon, S.
Thai viruses	Thailand	1988–1997	Parchariyanon, S.
38-96, 14-97, 33-97	Cuba	1996/1997	Sobrino, F.

1998; Parchariyanon et al., 1998; Björklund et al., 1999) and/or is derived from whole genome data available in Genbank. New data from Taiwan, Korea and Ukraine was acquired using standard techniques for amplification and sequencing of viral nucleic acids. Details on the sources of these viruses are provided in Table 1.

## 2.2. Standardisation of genetic typing

Table 2 summarises published phylogenetic studies on CSFV. The three regions of the CSFV genome that have been most extensively used for phylogenetic analyses are: (1) a 150 nt stretch of the 5' NTR (Greiser-Wilke et al., 1998); (2) a 190 nt stretch of the E2 gene (Lowings et al., 1996); and (3) a 409 nt stretch of the NS5B gene (Björklund et al., 1999), as shown in Fig. 1 and Table 3. This study therefore sets out to compare the utility of using these three regions for genetic typing, as well as to look at the overall extent and distribution of CSFV diversity. The number of viruses that have been sequenced is approximately 400 for each of the 5'NTR and E2 regions and 106 for the NS5B region.

A standardised approach to the generation of dendrograms that display genetic relatedness would simplify training of staff to undertake such work and would further improve interpretation and comparisons of results produced in different laboratories. We therefore selected a reproducible method for constructing phylogenetic trees from data sets of sequences so that trees with similar topographies should be produced with the same data regardless of the order of data entry. A summary of the methodology is given in Table 4. It was applied to each of the above three individual genomic regions, and also with all of the sequences combined into a single data set. Although the total amount of CSFV sequence data is now large, there were only 55 CSFV isolates for which all three regions had been sequenced. Included in these sequences were representatives of the main European groups as well as the disparate Congenital Tremor and Kanagawa viruses and newly sequenced Korean viruses (Table 5).

Table 2  
Published phylogenetic analyses of CSFV

Region of genome	Nucleotides sequenced number (position <sup>a</sup> )	Viruses sequenced number	Publications
5'NTR	150 (200–349)	88; 14; 8 <sup>b</sup>	Greiser-Wilke et al., 1998; Bartak and Greiser-Wilke et al., in press; Greiser-Wilke et al., in press
5'NTR	115 (247–362)	48	Hofmann et al., 1994
5'NTR	279 (99–378)	29	Stadejek et al., 1996
5'NTR	232 (132–363)	34	Stadejek et al., 1997
5'NTR	242 (121–362)	10; 37	Vilcek et al., 1997; Sakoda et al., 1999
5'NTR	355 (22–376)	78	Hofmann and Bossy, 1997; Hofmann, personal communication
5'NTR	321 (between 24 and 349)	24	Widjoatmodjo et al., 1999
E1/E2	475 (2345–2819)	8	Lowings et al., 1994
E1/E2	813 (2225–3038)	24	Widjoatmodjo et al., 1999
E2	190 (2518–2707)	112; 12; 14; 4 <sup>b</sup>	Lowings et al., 1996, 1999; Vilcek and Paton, 1998; Greiser-Wilke et al., in press
E2/NS2	254 (3408–3661)	76; 34	Vilcek et al., 1996; Stadejek et al., 1997
NS2	415 (4253–4667)	34	Stadejek et al., 1997
NS23	177 (5342–5518)	9	Harding et al., 1996
NS5B	212 (11577–11788)	8	Lowings et al., 1994
NS5B	207 (11360–11566)	32	Vilcek et al., 1996
NS5B	409 (11158–11566)	106	Björklund et al., 1999
NS5B/3'NTR	313 (11940–12252)	8	Vilcek and Belák, 1997
3'NTR	296–310 (11953–12249)	13	Björklund et al., 1998
3'NTR	186–191 (12068–12255)	36	Vilcek et al., 1999

<sup>a</sup> Nucleotide positions (where known) are given according to the sequence of Alfort 187 (Ruggli et al., 1996).

<sup>b</sup> Additional unpublished sequence data bring the total of viruses sequenced for these two regions up to approximately 400 each.

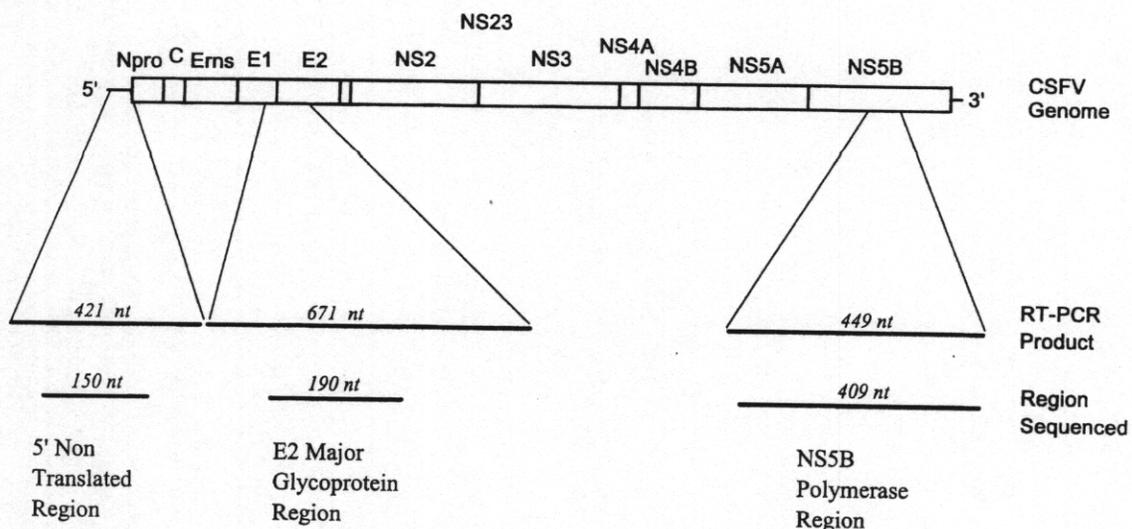


Fig. 1. Schematic representation of the CSFV genome showing the three selected RT-PCR target regions. The larger bars refer to the regions amplified by PCR and the smaller ones beneath represent the actual regions sequenced.

Table 3  
RT-PCR used in sequencing studies<sup>a</sup>

5' NTR		
Primers used for amplification of a 421 nt fragment of the 5' NTR of CSFV		
CSFV-UP1 (sense):	5' CTA GCC ATG CCC WYA GTA GG 3'	(94–113)
CSFV-UP2 (antisense):	5' CAG CTT CAR YGT TGA TTG T 3'	(514–496)
Internal Cy5 labelled primers for sequencing 150 nt		
CSFV/SQ-1 (sense):	5' AGC TCC CTG GGT GGT CTA 3'	(146–163)
CSFV/SQ-2 (antisense):	5' TGT TTG CTT GTG TTG TAT A 3'	(417–399)
E2 gene		
Primers used for amplification of a 671 nt fragment of E1 and E2 of CSFV		
Forward 5'	AGR CCA GAC TGG TGG CCN TAY GA 3'	(2228–2250)
Reverse 5'	TTY ACC ACT TCT GTT CTC A 3'	(2898–2880)
Internal primers for sequencing 190 nt of E2		
Forward 5'	TCR WCA ACC AAY GAG ATA GGG 3'	(2477–2497)
Reverse 5'	CAC AGY CCR AAY CCR AAG TCA TC 3'	(2748–2726)
NS5B gene		
Primers used for amplification of a 449 nt fragment of NS5B of CSFV		
S1 Forward 5'	GAC ACT AGY GCA GGC AAY AG 3'	(11138–11157)
S2 Reverse 5'	AGT GGG TTC CAG GAR TAC AT 3'	(11586–11567)
The same primers (S1 & S2) used for sequencing 409 nt of NS5B		

<sup>a</sup> Figures in parenthesis correspond to nucleotide positions in Alfort-187, Ruggli et al., (1996).

Each virus sequence was stored as text in the microsoft notepad program from whence it can be directly imported into the ClustalW program in order to carry out multiple sequence alignments (Thompson et al., 1994). Once aligned, the file is converted into Phylip format and used in the Puzzle4 program to estimate the transition–transversion (Ts–Tv) ratio of each data set (Strimmer and von Haeseler, 1996, 1997). The Ts–Tv ratios for the 5'NTR, E2, NS5B and all three regions combined were calculated as 2.8, 7.6, 6.9 and 6.2, respectively. The data sets were next analysed by DNAdist producing distance

Table 4  
Methodology for generation of phylogenetic trees

Action sequence	Program used	Notes
Sequence storage	Microsoft Notepad	
Sequence alignment	ClustalW	
Ts–Tv ratio calculation	Puzzle4	
Distance matrix calculation	DNAdist (Phylip)	Maximum likelihood method Set Ts–Tv ratio
Treelfile generation	Neighbor (Phylip)	Use multiple jumbles CSFV Kanagawa as outgroup
Tree drawing	Drawtree/Drawgram (Phylip)	
Statistical evaluation	Seqboot (Phylip)	
Phylogeny estimate	DNAdist (Phylip) Neighbor (Phylip)	Multiple data sets option=100 Multiple data sets option=100
Bootstrap values	Consense (Phylip)	CSFV Kanagawa as outgroup

Table 5  
 Virus sequences taken from Hannover database for use in '55 CSFV data-set' phylogenetic analysis

Catalogue No.	Original name	Year of isolation	Country	Host
CSF0007	V 744	1984	D	dp <sup>a</sup>
CSF0008	V 750	1984	D	dp
CSF0014	Sch 180	1989	D	dp
CSF0062	Vi 633/90	1990	D	wb <sup>b</sup>
CSF0074	SP 2087/90	1990	A	dp
CSF0084	RostockII, V119/92	1992	D	dp
CSF0106	Celle/Han94, V422	1994	D	dp
CSF0107	V 1166 III/94	1994	D	wb
CSF0120	SP 4165/23	1994	A	wb
CSF0126	Kaernten/95; 933	1995	A	dp
CSF0130	Switzerland IV/93	1993	CH	–
CSF0166	EWS 1053/96	1996	D	wb
CSF0171	NWS 718/96	1996	D	wb
CSF0173	LN-SLOW	–	SLO	–
CSF0265	Vi861/863/97/OL	1997	D	wb
CSF0273	V3/97	1997	D	dp
CSF0277	V1240/97	1997	D	dp
CSF0281	Vi302/97/OL; Emstek	1997	D	dp
CSF0283	MP104	1997	NL	dp
CSF0290	36/5	199?	PL	dp
CSF0292	25/5	199?	PL	dp
CSF0294	29/5	199?	PL	dp
CSF0295	26/5	199?	PL	dp
CSF0297	14/2	1992	PL	dp
CSF0299	19/2	1992	PL	dp
CSF0302	4905 I 97/03	1997	I	dp
CSF0303	4917 I 97/04 SA	1997	I	dp
CSF0305	VRI 2277	1986	MAL	–
CSF0306	VRI 4167	1986	MAL	–
CSF0307	EVI 192	1987	BR	–
CSF0308	1825	1986	B	–
CSF0309	Kanagawa (Tap3)	1974	JP	–
CSF0310	c4D (N8)	1991	I	–
CSF0311	n6W (N3)	1992	I	–
CSF0316	7/1	1991	PL	dp
CSF0317	2/1	1991	PL	dp
CSF0318	2/4	199?	PL	dp
CSF0320	4/6	199?	PL	dp
CSF0410	Congenital Tremor	1964	GB	–
CSF0902	Alfort 187	1968	F	–
CSF0905	Brescia	1945	I	–
CSF0906	Bergen	1977	NL	–
CSF0910	Eystrup	1964	D	–
CSF0919	ALD	1964	JP	–

<sup>a</sup> dp: Isolated from a domestic pig.

<sup>b</sup> wb: Isolated from a wild boar.

matrices derived from the number of nucleotide substitutions between the sequences (Felsenstein, 1989). In DNAdist, the maximum likelihood method of evolution was chosen and the estimated Ts–Tv ratio was set to the above-calculated values. The output from DNAdist was then used as the input file in a neighbor-joining tree generating method, in this case Neighbor. Since the topology of the trees becomes more complex as the number of viruses increases, multiple jumbles ( $\times 99$ ) were used and the outgroup was always specified as the sequence of the Kanagawa virus. This ensures that the tree will have a similar topography each time it is drawn. The treefile generated in the Neighbor program was then used as the input for a tree drawing program, namely; drawtree or drawgram although the trees can be looked at using other programs such as Treeview (Page, 1996).

To assess the statistical reliability of the dendrograms produced, each data set was repeatedly reanalysed using the Seqboot program. As before, the phylogeny for each repeat was calculated using DNAdist and then Neighbor. Both programs were run as described above except that the multiple data sets option (M) was set to 100 to indicate how many replicates of the alignment were in the input file. The treefile from Neighbor was then used as the input file in the Consense program, again designating the outgroup as the Kanagawa sequence. The bootstrap values were then readable in a text format which could be directly correlated to the dendrogram. Values in excess of 70% were considered to be significant (Clewley, 1999).

The nomenclature of Lowings et al. (1996) was used as a starting point to describe virus groups and subgroups.

### 2.3. Overall diversity of CSFV

The most representative sequence data is available for the E2 and 5'NTR regions. Since the former gives better discrimination, it was used to examine the overall diversity of CSFV. One hundred sequences from viruses representative of all so far described subgroups were aligned and compared using the above-described methodology. Twenty-four of these viruses had also been analysed in the above-mentioned '55 virus data-sets'. The viruses included 16, 12, 9, 12, and 24 known representatives from the groups defined by Lowings et al., (1996) — 1.1, 1.2, 2.1, 2.2, and 2.3, respectively. Additionally, recent sequence data were included from the Ukraine, Taiwan, Korea, and Thailand as well as the two known disparate viruses (Kanagawa and Congenital Tremor).

## 3. Results

### 3.1. Phylogenetic studies

The phylogenetic tree comparing the 55 CSFVs using the E2 sequence data-set (190 nt) is shown in Fig. 2a. Many of the sequences were the same ones analysed by Lowings et al. (1996), and therefore the nomenclature of Lowings is readily assigned. The new Korean isolates appear as a separate group, and the distinctive nature of the Kanagawa and Congenital Tremor viruses is evident. The NS5B (409 nt) and 5'NTR (150 nt) trees

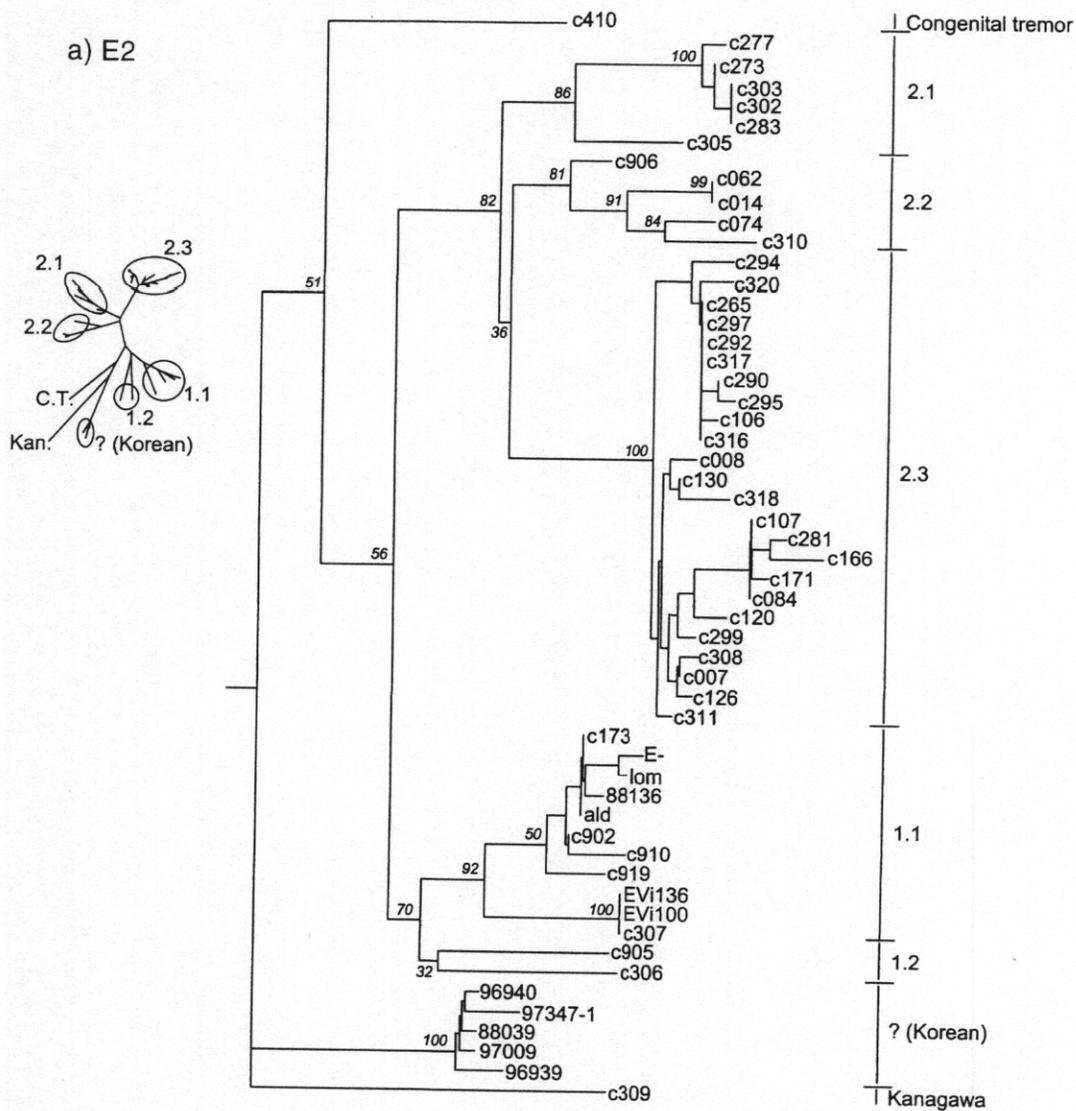


Fig. 2. Phylogenetic trees obtained with sequence data from 55 CSF viruses, based on analysis of (a) the 190 nt E2 sequence data-set; (b) the 409 nt NS5B sequence data-set; (c) the 150 nt 5'NTR sequence data-set. Horizontal branch lengths are proportional to genetic distances between isolates. A mini-view of the same data analysis expressed as a spider dendrogram (all branch lengths are proportional to genetic distances) is shown to the left of each tree. Virus identities are those used in the database of the EU Reference Laboratory for CSF (Greiser-Wilke et al., this issue) or else are described in Table 1. Bootstrap figures are shown in italics for the major nodes. Names of virus subgroups are based on the nomenclature of Lowings et al. (1996).

(Fig. 2b and c) have essentially the same clusters. Group 2 and its three subgroups are the same in all three trees, as is the separate grouping of a cluster of recent Korean viruses and the Kanagawa virus. However, the confidence levels are highest for the NS5B tree and lowest for the 5'NTR. The position of the Congenital Tremor virus (c410) varies between trees, but the bootstrap values for its node are high only for the NS5B tree, which is therefore the most likely to be correct. The same problem arises with subdivision within Group 1, where considerable diversity is evident, but the positions of the viruses c905 and c306 are variable but predicted with low confidence. Interpretation of fine

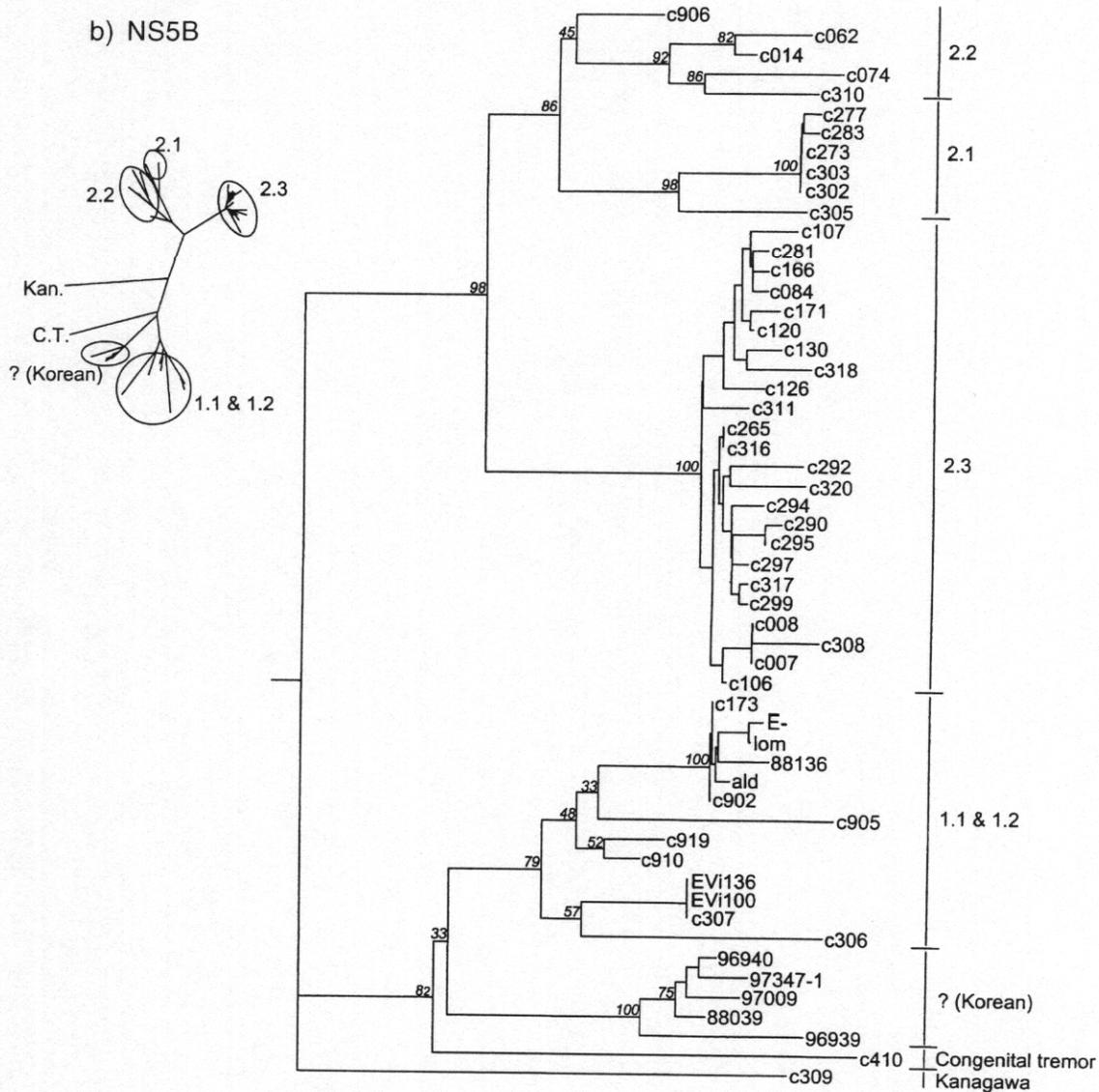


Fig. 2. (Continued).

changes within a subgroup is less reliable due to random events. For example, within Subgroup 2.1, all viruses other than c305 are identical with respect to 5'NTR sequence, but c277 and c273 are distinct in the E2 region, whereas c277 and c283 are distinct in the NS5B region.

A phylogenetic tree was also produced based upon summated sequence data from all three regions (749 nt, data not shown). The topography of this tree was very similar to that of the NS5B data, except that the positions of the c905 and c306 viruses are more similar to the E2 tree. Bootstrap values for the combined data set tree are marginally higher than for the NS5B tree in most but not all nodes.

In Fig. 3, a phylogenetic tree is shown using the same 190 nt E2 region as in Fig. 2a, but incorporating 100 rather than 55 CSFV isolates. Group 2 and its three subgroups are all predicted with a high degree of confidence, although the node point from which the

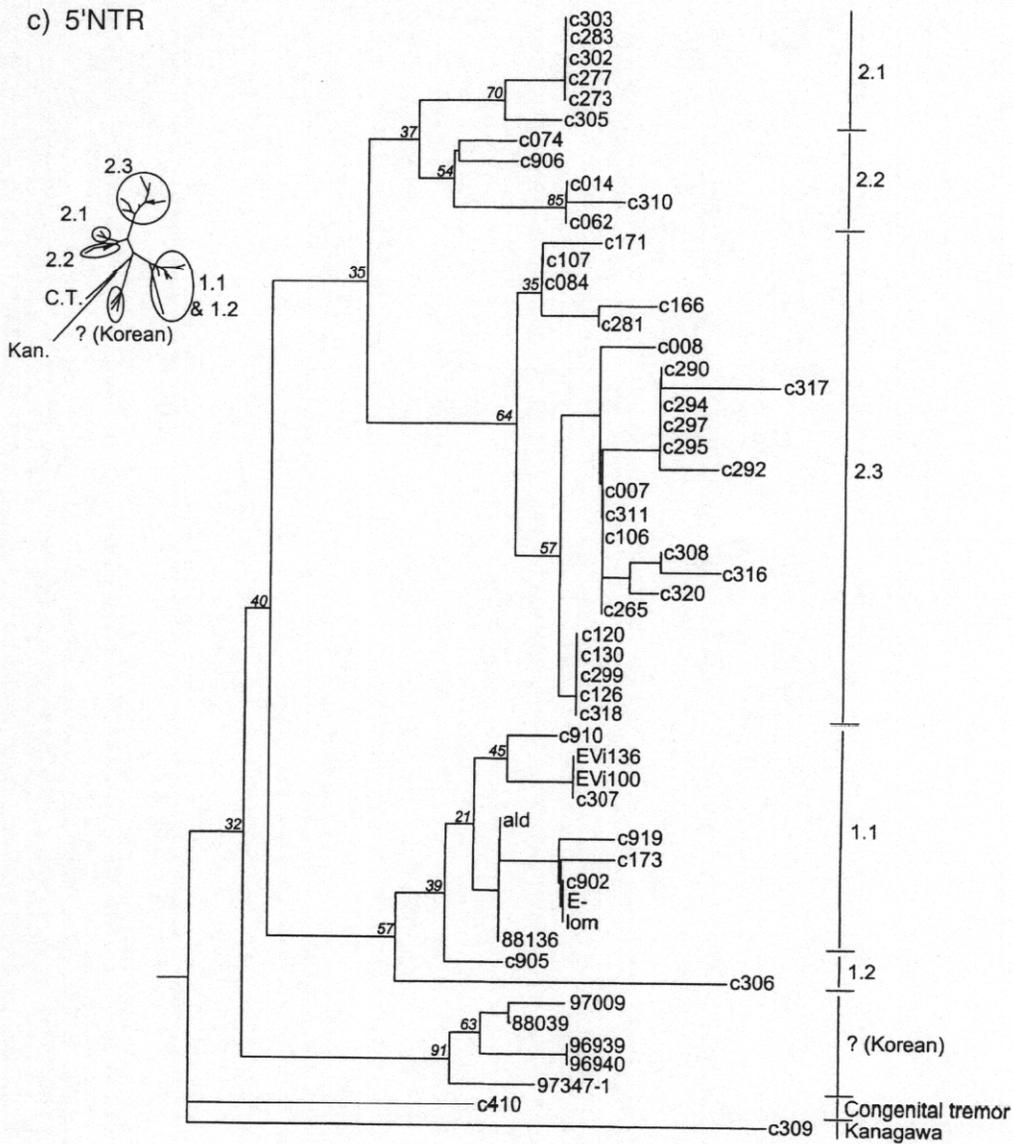


Fig. 2. (Continued).

subgroups diverged is still uncertain. Group 1 is a highly divergent collection of viruses, but the low bootstrap values for the different clusters makes it difficult to decide on a definite classification. There are three main subgroups evident, which can be only tentatively assigned as 1.1, 1.2, and 1.3. The old US and UK viruses from the 1940s, 1950s and 1960s form the basis of the Subgroup 1.1 as defined by Lowings et al. (1996). Subgroup 1.2 consists of the historic Brescia and Baker A strains from Italy and US, recent Ukrainian isolates, the VRI isolates from Malaysia, a New South Wales virus, and the viruses 14-97 38-96 and 33-97 from Cuba. Subgroup 1.3 includes the CBR/91, BKK/88, VRI2658 and VRI4167 isolates from Thailand and Malaysia, and a highly divergent isolate from Honduras. Three separate clusters are confidently predicted for viruses from Korea, Thailand, and Japan/Taiwan. The position of the historic Congenital Tremor strain from UK remains uncertain.

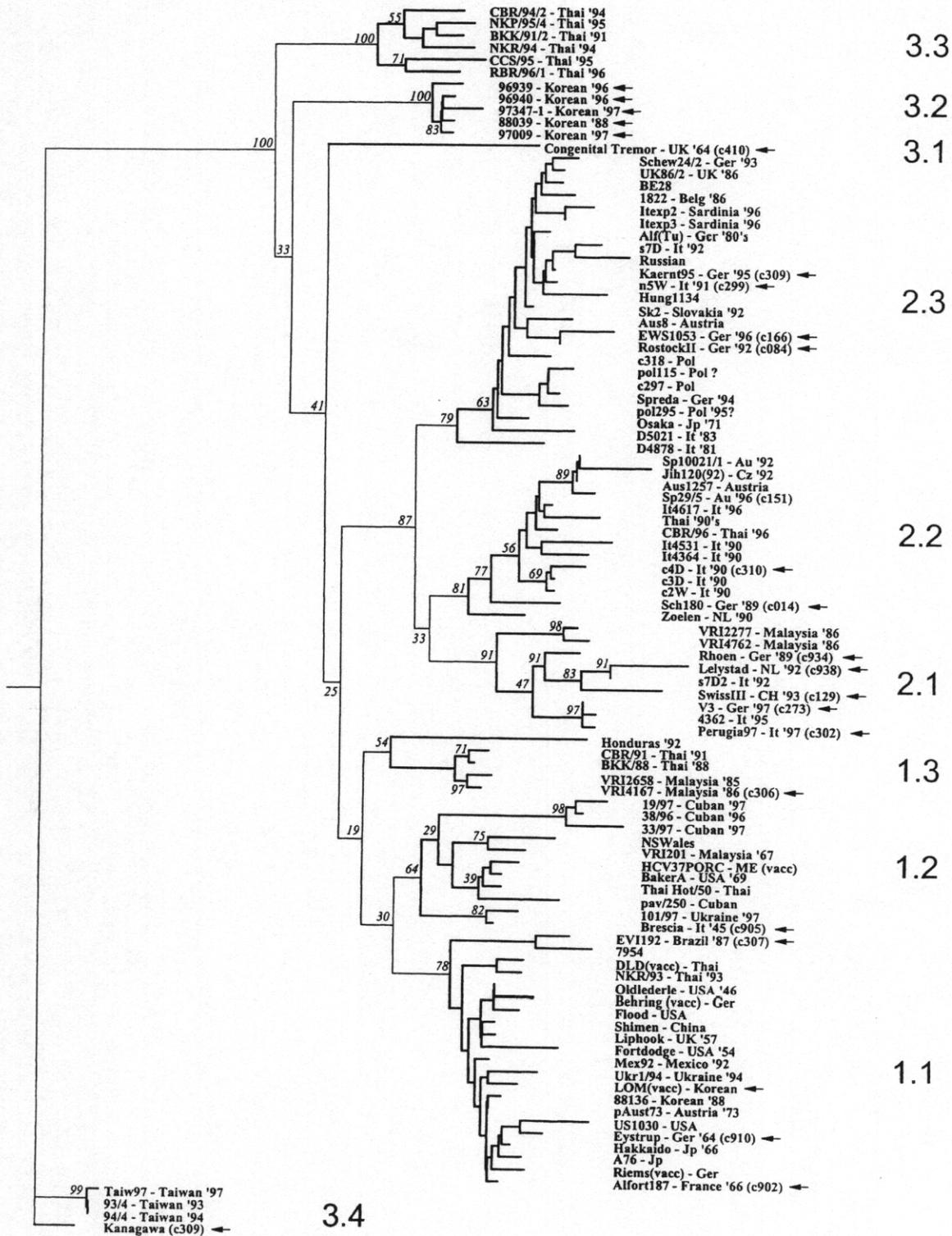


Fig. 3. Phylogenetic tree obtained with sequence data from 100 diverse CSF viruses, based on analysis of the 190 nt E2 sequence data-set. Horizontal branch lengths are proportional to genetic distances between isolates. Viruses identified are (a) those in common with Fig. 2 — given original name and Hannover database number and arrowed; (b) The other virus names are explained in Table 1. Bootstrap figures are shown in italics for the major nodes.

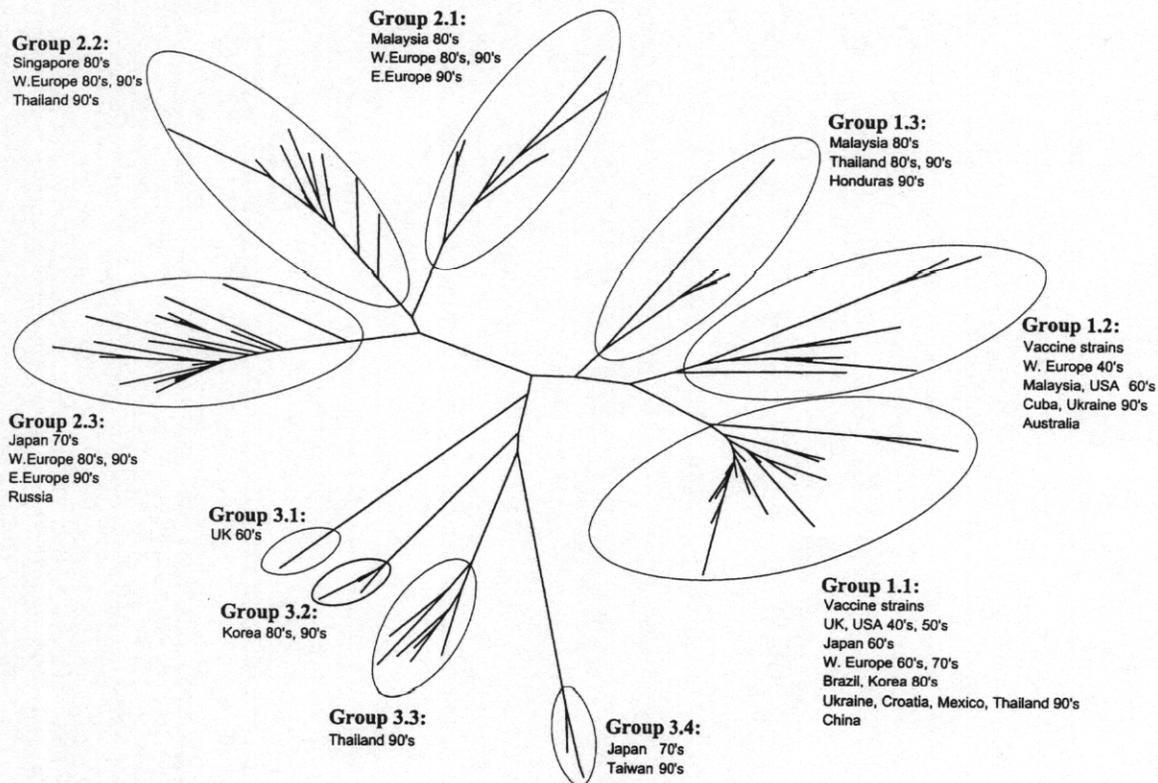


Fig. 4. The extent of CSF virus diversity as revealed by a phylogenetic tree obtained with 190 nt of E2 sequence data from 100 CSF viruses. The phylogenetic analysis is the same as was used to generate Fig. 3, but the tree is shown in a different format such that all branch lengths are proportional to genetic differences. Virus subgroups are assigned based on the phylogenetic analysis and are based on the nomenclature of Lowings et al. (1996).

### 3.2. The geographic distribution of CSFV diversity

The world-wide occurrence of CSFV is dealt with in a previous chapter (Edwards et al., this issue). Unfortunately, isolates are not available for study from all regions where CSFV occurs or has occurred. A simplified version of Fig. 3 to show an international comparison of CSFV isolates is illustrated in Fig. 4. Phylogenetic analysis of the sequences was based on the 190 nucleotide region of E2 as described above. Inspection of the phylogenetic tree suggests that as well as the two groups described by Lowings et al. (1996) an additional subgroup to Group 1 (1.3) is evident along with a third group divided into four subgroups. On the basis of Fig. 4 and taking into account other studies of CSF virus distribution (Table 2), the following summary can be made.

#### 3.2.1. Europe

The small number of available isolates from the period between 1920 and 1970 are all of Group 1, except for the highly distinctive Congenital Tremor strain which was isolated in Britain in the 1960s. Most of the isolates from this period are of Subgroup 1.1, similar to Alfort 187, which is a reference strain widely used in diagnostic serology. An example

of an early Subgroup 1.2 is the Brescia virus from Italy. Most if not all modified live vaccines are also thought to derive from Subgroup 1.1 or 1.2 isolates made in this period. No Subgroup 1.3 viruses have been identified in Europe. Since 1970, isolations of Type 1 viruses have been very infrequent in Europe, exceptions being a Belgian isolate from 1989 (isolate Basavelde a Type 1.1; Vanderhallen and Koenen, 1997) and Ukrainian isolates from the 1990s with representatives from both Subgroups 1.1 and 1.2.

Virtually all of the large number of isolates made in the 1980s and 1990s have been of Group 2. The earliest appearance of Group 2.3 viruses was in Germany in 1982. Subsequently these viruses have been found in many different countries, up to the present time, including Italy, Sardinia, France, Belgium, Britain, Austria, Switzerland, Hungary, The Czech Republic, Poland, and The Slovak Republic. Four principal sub-subgroups have been recognised by genetic typing based on 5'NTR sequence comparisons (Greiser-Wilke et al., 1998). The reliability of this fine discrimination is confirmed by typing with monoclonal antibodies (Kosmidou et al., 1995). CSF viruses of Types 2.2 and 2.1 have been more restricted in their distribution. The 2.2 viruses have been found in Central Europe, in Austria, The Czech Republic, Italy, Germany, Romania and Hungary, in the period from 1985 onwards. Viruses of both the 2.2. and 2.3 subgroup have been isolated repeatedly from wild boar in different parts of Europe.

The 2.1 viruses had been only sporadically reported in Europe until their involvement in a recent major epizootic in 1997. They have not been isolated from European wild boar. The first European isolate was recorded in Germany in 1989, and thereafter in the Netherlands (1992), and Switzerland (1993). A 2.1 subgroup virus was also discovered as a contaminant of wild boar meat imported from China into Austria in 1993. During 1997 and 1998, the virus is believed to have been introduced from Germany into the Netherlands, with subsequent spread to Italy, Belgium and Spain (Widjoatmodjo et al., 1999; Greiser-Wilke et al., in press).

### 3.2.2. America

Only Type 1 viruses have so far been reported. Historic isolates are only available from North America and it is notable that the British and North American isolates from the 1940s and 1950s are similar Type 1.1 viruses. Type 1.1 isolates have also been identified from Brazil in the 1980s and from Mexico in the early 1990s. A virus from Honduras, tentatively placed in Subgroup 1.3, is rather distantly related to other Type 1 CSF viruses. Recent outbreaks of CSF in Cuba have been associated with viruses of Type 1.2 (de Arce et al., 1998).

### 3.2.3. Asia

All of the major genetic groups and subgroups of CSFV have been identified in different parts of Asia at different times.

Historic isolates are available from Japan. The Japanese Hokkaido virus from 1966 is of Type 1.1, whereas the later Osaka isolate from 1971 and the Kanagawa isolate from 1974, are the earliest available representatives of Subgroup 2.3 and 3.4, respectively.

In the 1980's, Subgroup 1.1 viruses were found in Thailand, and at the same time Subgroup 1.2 viruses were found in both Thailand and Malaysia (Lowings et al., 1996; Parchariyanon et al., 1998). Subgroup 2.1 viruses were also found in Malaysia in the

1980s. A Subgroup 2.1 virus was isolated from wild boar meat imported into Austria from China as described above. Subgroup 2.2 viruses have been found in Thailand in the 1990s.

Two new groups of viruses (3.2 and 3.3) have been identified in Korea and Thailand, respectively.

### 3.3. *The spread and persistence of CSFV*

Since few or no isolates are available from many countries that have experienced recent or historical infection, conclusions on the world-wide origin and spread of the different CSFV variants remain tentative. In some regions there appears to have been a marked change in the predominating variant. For example, in Europe there has been a switch from Group 1 viruses prior to the 1970s, to Group 2 viruses thereafter. This might indicate that attempts to control and eliminate the disease were thwarted by reintroduction of virus from elsewhere, with Group 2.1 viruses representing the newest introduction of all. However, the historical isolate collection is too small to be certain that Group 2 viruses were not already present in parts of Europe prior to the 1980s. Elsewhere, such as in Central America, Group 1 viruses appear to have persisted for many years. The greatest diversity of isolates appears to be present in Asia, even though there are only five countries from which isolates have been sequenced. The Group 3.2, 3.3 and 3.4 variants have not been found anywhere else.

## 4. Discussion

It is important to harmonise genetic typing methods so that the sequence data obtained in different laboratories are comparable. Providing a large collection of sequences from one or more specific target regions increases the likelihood of such regions being used in future studies also. The work of Hofmann et al. (1994), Lowings et al. (1996) and Björklund et al. (1999) had already generated much data for the 5'NTR, E2 and NS5B regions. Lowings et al. (1996) had compared E2 (190 nt) and 5'NTR (96 nt) sequence data for a common set of CSF viruses and showed that although the two methods gave similar subdivisions the more variable E2 region gave better discrimination. In this paper we report a comparison between sequence data from a slightly extended 5'NTR region and the E2 and NS5B regions as described by Lowings et al. (1996) and Björklund et al. (1999).

It has been shown for Hepatitis C virus another member of the Flaviviridae family, that essentially similar viral subdivisions can be obtained by sequence comparisons derived from many different target regions of the genome (Simmonds et al., 1996). Each of the three regions reported here segregate CSFV isolates into similar clusters. The fact that this is so, indicates that recombination between the genomes of viruses from within different genetic clusters has been uncommon or has not occurred. As a phylogenetic target, the 5'NTR is highly conserved, so that the PCR primers used for its amplification can also readily be applied to other pestiviruses, enabling sequence comparisons to be made across the genus. This genetic stability also makes the region a favourite target for

diagnostic PCR where a broad specificity of viral recognition is important. The need to be assured of the efficacy of these diagnostic PCR primers is indeed one reason for continuing to sequence viruses in this region. However, 150 nt is only a small amount of sequence to use from a conserved region of the genome and this leads to poorer discrimination within the CSFV genotype than can be obtained with the other two regions, and in a lower confidence in phylogenetic predictions. Sequencing a bigger portion of the 5'NTR has been shown to give better discrimination between CSF viruses (Hofmann and Bossy, 1997).

In practice, sequence data from this region of the 5'NTR have been used as a tool to trace the spread of CSF outbreaks in Germany (Greiser-Wilke et al., 1998; Fritzscheier et al., in press) and in different countries of Europe between 1997 and 1998. The sub-subgrouping of 2.3 viruses from Germany appeared to be consistent with the known epidemiology, despite a low level of confidence by bootstrapping in the phylogenetically based subdivisions. In this case, the genetic differences between the sub-subgroups were between 3 and 9%. When the same method of typing was applied to viruses from the recent 2.1 epizootic, very few nucleotide changes (1–2) were evident and the groupings that were predicted (confidence levels between 32 and 66%) were inconsistent with the chronology of events. In contrast, analysis based on the E2 sequence clearly allowed the distinction between one cluster of isolates from previous outbreaks (1989 and 1993), and a second cluster comprising the isolates from the 1997–1998 epizootic. The confidence level for these two clusters was of 99% (Greiser-Wilke et al., in press). Epidemiological data are only available for one of the older isolates, which had been introduced to Austria by an illegal import of wild boar meat from China (Hofmann and Bossy, 1998). Phylogenetic analysis indicates that this isolate is in different clusters in both cases. In addition, the derived amino acid sequences indicate that all the viruses in Subgroup 2.1 — excepting the Lelystad isolate (1992) — are very similar or even identical.

The discrimination between isolates is similar using the 190 nt E2 target region or the 409 nt NS5B target region. Of the 55 isolates compared, 11 pairs appear identical in sequences from the E2 region, compared to eight from the NS5B region. The most divergent viruses had percentage nucleotide identities of 78.9 and 83.4 for E2 and NS5B regions respectively. By comparison the figures for the 5'NTR are 32 pairs of identical isolates and a maximum divergence of 12%. In considering the classification of CSFV and the assignment of viruses to groups and subgroups, the NS5B data gives the most certain phylogenetic classification of the three regions examined, with the highest bootstrap values for its branch nodes. The fact that this is so, despite the greater variability of the E2 region may be due to a higher frequency of back-mutations in the E2 region which confuses evolutionary analysis. It is also to be expected that finer and more accurate discrimination will be achieved when more sequence is analysed (Simmonds et al., 1996). However, a combined data-set based on the sequence from all three CSFV regions resulted in only marginal improvement in statistical confidence compared to using the NS5B data alone. The accuracy of phylogenetic predictions also depends on the number and representativeness of the isolates analysed, and the accuracy improves as more sequences are included.

Although the grouping of isolates is essentially the same with the three genomic regions selected and generally coincides well with epidemiologic findings, the method is

still hampered by the low confidence levels achieved. In such circumstances, and especially if the results conflict with epidemiological features such as the locality or chronology of isolation, it is reassuring if the same typing is achieved from two different genomic regions. This is particularly the case when comparing very similar viruses, where there is otherwise a risk of over interpreting small differences that may amount to background noise, or even PCR induced errors. The latter can be minimised by pooling amplicons from several reactions prior to sequencing, or by using a polymerase with proof reading capability. It is also advisable to study different genomic regions whenever new isolates are included in the analyses that fall into new or distinct clusters, but with low (<70%) bootstrapping values. Although, depending on the amount of additional sequence generated, this may not always resolve the uncertainty over the phylogenetics, it should confirm that the virus in question is unusual.

A definitive definition of groups and subgroups cannot be established on a convincing mathematical basis, without more conclusive bootstrap results. Direct pairwise sequence comparisons were also compared with phylogenetic analysis for the segregation of viruses into related clusters (data not shown). The best agreement was obtained with the NS5B data-set, where the frequency distribution of pairwise identities suggested a subdivision at three hierarchical levels (groups, subgroups and isolates) with boundaries at 89.5 and 95.5% identity. In other words, viruses with nucleotide identities of greater than 95.5% are defined as isolates within the same subgroup; identities of between 89.5 and 95.5% put viruses in the same group but different subgroups, whilst identities of less than 89.5% put viruses into different groups. Concordance between this method of classification and clusters defined by phylogenetic trees was not perfect. This is a common problem, and in general, phylogenetic predictions are expected to be more accurate (Simmonds et al., 1996). Also problematic is the fact that the relationships change as more virus sequences are considered, and therefore any system of nomenclature can only be considered transitional. Nevertheless, some system is necessary in order to discuss the pattern of variation that can be observed. From Figs. 2 and 3, it can be seen that the nomenclature of Lowings et al. (1996) requires revision to accommodate additional groups and subgroups. From Fig. 2 it can be seen that the previously described Subgroups 2.1, 2.2, and 2.3 can be defined with statistical probabilities of greater than 70%. The diversity within Group 1 justifies subdivisions, but with the small number of viruses analysed in this data-set, it is not certain how these subgroups should be formed. The Congenital Tremor, Kanagawa and Korean viruses represent either new groups or subgroups.

When a larger E2 region data-set is examined that is more representative of the total diversity of CSFV then the largest number of groups or subgroups is apparent. A tentative assignment based on analysis of Fig. 4 and adapting rather than overturning the previous nomenclature would be 1.1, 1.2, 1.3, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, 3.4.

Although genetic diversity is evident, it is important to keep in mind that all CSFVs are considered to be closely related antigenically, and none have been shown to lack the highly conserved neutralisation epitope on the major envelope glycoprotein (Paton, 1995). For the 100 virus E2 region data-set, the lowest nucleotide identity between two CSFV isolates was 72% (Taiwan-P97 and German-Eystrup). This compares to 53, 44 and 55% identity for the same genomic region comparing CSFV Alfort Tubingen and BVDV I

(NADL), BVDV II (890) and BDV (BD31), respectively. Nevertheless, further studies to confirm the antigenic relationship between these different genetic clusters of CSFV would be desirable.

Consideration of historic changes in predominating virus types must be made with great caution, because only small numbers of isolates are available and it cannot be relied upon that they are representative of the full picture. Obtaining a truly representative set of viruses and sequences should make disease tracing more effective in the future. The greatest numbers of pigs are to be found in China, and it is disappointing that so few CSFV isolates are available from this country. Looking at the distribution of CSFV types on a world-wide basis, it is notable that the greatest diversity is found in the Far East. All the major groups and subgroups have been found here at some time, and it is tempting to speculate that this could be where CSFV originated, despite the fact that early reports of the disease are divided between a possible new and old world origin (Anon, 1962).

In Europe there seems to have been a change from Group 1 viruses which predominated in the 1940s and 1950s, to Subgroup 2.2 and 2.3 viruses in the 1980s. Unfortunately, there are few isolates available from the intervening years of the 1950s, 1960s and 1970s, but the Alfort 187 strain from France in 1966 is in Subgroup 1.1. The earliest available isolate of Subgroup 2.3 is from the Far East (Japan, Osaka 1971). If there was indeed a shift in Europe from Group 1 viruses to Group 2 viruses, during the 1970s, then it is not clear why this occurred. Interestingly, a small number of Group 1 viruses have recently been identified in Europe from Belgium (Basevalde 1989) and Ukraine (1990s), indicating that they may not have been completely eliminated, that new introductions have occurred, or that they represent vaccine 'escapes'. By contrast, in America only Group 1 isolates were made up to the point of the disease's eradication in the 1970s, whilst isolations from central America and the Caribbean have also been of this group.

Viruses of the Subgroup 2.1 do not appear to have originated in western Europe, but rather to have emerged sporadically since 1989, culminating in the major epizootic of 1997/1998 (see chapter in this volume). The only other places these viruses have been found is in Malaysia (1986) and China (1993) and this could be an indication of an Asian origin for this subgroup.

In the far East, viruses of the Kanagawa type have clearly been present for many years since the original Japanese isolate was made in 1974, and similar viruses have been identified in Taiwan in the 1990s. In Thailand and Korea, the diversity of the Group 3.2 and 3.3 viruses would be consistent with their having been present for many years. None of these viruses have so far been reported in Europe or America.

A number of phylogenetic studies have compared viruses from geographically restricted outbreaks and found that many of them are extremely similar. For example, Vilcek and Paton (1998) studied Subgroup 2.3 CSFVs from the Slovak Republic and found that isolates obtained as much as 4 years apart differed by only a single nucleotide in the 409 nt NS5B region. Similarly, isolates from the European epizootic of 1997/8 involving Subgroup 2.1 viruses, were found to differ from one another by at most only a very small number of nucleotide substitutions in the 5'NTR or E2 regions (Widjoatmodjo et al., 1999; Greiser-Wilke et al., in press). Experimental studies have also shown that different parts of the CSFV genome are stable when passaged in cell

cultures and in pigs (Vanderhallen et al., 1999). This implies that on the one hand large regions must be sequenced to differentiate closely related viruses, whilst on the other, even small differences in sequence can have epidemiological significance. This further supports the use of a data-set combining all three of the selected regions in order to achieve a higher level of discrimination. This approach was shown to be the most accurate when tracing the spread of similar CSFV isolates in Central Europe, using three different genomic targets (Stadejek et al., 1997).

## 5. Conclusions

We have analysed multiple strains of CSFV from three genomic regions and assessed their ability to be used in phylogenetic analysis and their use in classifying the strains. All three regions grouped the viruses very similarly although there were slight discrepancies in discriminating Subgroups 1.1 and 1.2 with the 5'NTR and NS5B sequences. Moreover, although the phylogenetic trees were very similar the statistical confidence separating the groups varied drastically and it was shown that the NS5B sequence data is the most useful single set. More viruses need to be sequenced in this region. When bootstrap values are low, it is useful to analyse more than one genomic region, in order to be confident of the validity of the genetic typing. Analysis of strains from the Far East demonstrated additional groups of CSFVs, one of which showed close evolutionary links with the previously disparate Kanagawa virus. It is hoped that the method presented here for the construction of phylogenetic trees with respect to CSFV will help to standardise future phylogeny and allow direct comparisons of results obtained in different laboratories.

## Acknowledgements

The work was supported by EU FAIR grant CT95-707. The work at VLA was also supported by UK MAFF grant SE0752.

## References

- Anon, 1962 History of hog cholera research in the US Department of Agriculture 1884–1960. Agriculture Information Bulletin No 241, Agriculture Research Service, US Department of Agriculture, Washington, DC, January 1962.
- Bartak, P., Greiser-Wilke, I. Genetic typing of CSF virus isolates from the territory of the Czech Republic. *Vet. Microbiol.*, in press.
- Björklund, H.V., Stadejek, T., Vilcek, S., Belák, S., 1998. Molecular characterization of the 3' noncoding region of classical swine fever vaccine strains. *Virus Genes* 16, 307–312.
- Björklund, H., Lowings, P., Paton, D., Stadejek, T., Vilcek, S., Greiser-Wilke, I., Belák, S., 1999 Phylogenetic comparison and molecular epidemiology of classical swine fever virus. *Virus Genes*, 19, 189–195.
- Clewley, J.P., 1999. A user's guide to producing and interpreting tree diagrams in taxonomy and phylogenetics Part 2. The multiple alignment of DNA and protein sequences to determine their relationships. *Communicable Disease and Public Health* 1, 132–134.
- de Arce, H.D., Frias, M.T., Barrera, M., Ganges, L., Nunez, J.I., Sobrino, F., 1998. Analysis of genomic

- differences between Cuban classical swine fever virus isolates. Poster presentation, OIE symposium on classical swine fever, Birmingham, United Kingdom, 9–10 July, 1998.
- Edwards, S., Fukusho, A., Lefevre, P., Lipowski, A., Pejsak, Z., Roehe, P., Westergaard, J. 2000. Classical swine fever: the global situation. *Vet. Microbiol.* 73, 103–119
- Felsenstein, J., 1989. Phylip phylogeny inference package (version 3.5c). *Cladistics* 5, 164–166.
- Fritzemeier, J., Greiser-Wilke, I., Depner, K., Moennig, V., The epidemiology of CSF in Germany between 1993 and 1997. Report on annual meeting of the national swine fever laboratories, Vienna, Austria, 16–17 June 1997, Document V1/7888/97, Commission of the European Communities pp. 33–35.
- Greiser-Wilke, I., Depner, K., Fritzemeier, J., Haas, L., Moennig, V., 1998. Application of a computer program for genetic typing of classical swine fever virus isolates from Germany. *J. Virol. Methods* 75, 141–150.
- Greiser-Wilke, I., Fritzemeier, J., Koenen, F., Vanderhallen, H., Rutili, D., De Mia, G.-M., Romero, L., Sanchez-Vizcaino, J.M., San Gabriel, A. Molecular epidemiology of a large classical swine fever epizootic in the European Community in 1997–1998. *Vet. Microbiol.*, in press.
- Greiser-Wilke, I., Zimmermann, B., Fritzmeier, J., Floegel, G., Moennig, V., 2000. Structure and presentation of a database in the World Wide Web of the CSF virus isolates held at the EU reference laboratory. *Vet. Microbiol.* 73, 131–136.
- Harding, M.J., Prud'homme, I., Gradil, C.M., Heckert, R.A., Riva, J., McLaurin, R., Dulac, G., Vydelingum, S., 1996. Evaluation of nucleic acid amplification methods for the detection of hog cholera virus. *J. Vet. Diagn. Investigation* 8, 414–419.
- Hofmann, M.A., Brechtbuhl, K., Stauber, N., 1994. Rapid characterization of new pestivirus strains by direct sequencing of PCR-amplified cDNA from the 5' noncoding region. *Arch. Virol.* 139, 219–229.
- Hofmann, M.A., Bossy, S., 1997. The 5'NTR of the classical swine fever virus genome is suitable for a detailed strain discrimination. Poster presented at the 4th International Congress of Veterinary Virology, Edinburgh, August 24–27, 1997.
- Hofmann, M.A., Bossy, S., 1998. Classical swine fever in 1993 in Switzerland: molecular-epidemiologic characterization of the virus isolate. *Schweiz. Arch. Tierheilkd.* 140, 365–370.
- Ishikawa, K., Nagai, H., Katayama, K., Tsutsumi, M., Tanabayashi, K., Takeuchi, K., Hishiyama, M., Saitoh, A., Gotoh, K., Muramatsu, M., Yamada, A., 1995. Comparison of the entire nucleotide and deduced amino acid sequences of the attenuated hog cholera vaccine strain GPE- and the wild-type parental strain ALD. *Arch. Virol.* 140, 1385–1391.
- Kosmidou, A., Ahl, R., Thiel, H.-J., Weiland, E., 1995. Differentiation of classical swine fever virus (CSFV) strains using monoclonal antibodies against structural glycoproteins. *Vet. Microbiol.* 47, 111–118.
- Lowings, J.P., Paton, D.J., Sands, J.J., De Mia, G.M., Rutili, D., 1994. Classical swine fever: genetic detection and analysis of differences between isolates. *J. Gen. Virol.* 75, 3461–3468.
- Lowings, P., Ibata, G., Needham, J., Paton, D., 1996. Classical swine fever diversity and evolution. *J. Gen. Virol.* 77, 1311–1321.
- Lowings, P., Ibata, G., De Mia, G.M., Rutili, D., Paton, D., 1999. Classical swine fever in Sardinia: epidemiology of recent outbreaks. *Epidemiol. Infect.* 122, 553–559.
- Meyers, G., Rümenapf, T., Thiel, H.J., 1989. Molecular cloning and nucleotide sequence of the complete genome of hog cholera virus. *Virology* 171, 555–567.
- Meyers, G., Thiel, H.-J., 1996. Molecular characterisation of pestiviruses. *Adv. Virus Res.* 47, 53–118.
- Moormann, R.J.M., Warmerdam, P.A.M., van der Meer, B., Schaaper, W.M.M., Wensvoort, G., Hulst, M., 1990. Molecular cloning and nucleotide sequence of hog cholera virus strain Brescia and mapping of the genomic region encoding envelope protein E1. *Virology* 177, 184–198.
- Moormann, R.J.M., van Gennip, H.G., Miedema, G.K., Hulst, M.M., van Rijn, P.A., 1996. Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. *J. Virol.* 70, 763–770.
- Paton, D.J., 1995. Pestivirus diversity (review). *J. Comp. Pathol.* 112, 215–236.
- Page, R.D.M., 1996. TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* 12, 357–358.
- Parchariyanon, S., Damrongwatanapokin, S., Inui, K., Lowings, P., Paton, D., Pinyochon, W., 1998. Different genetic spectrum of classical swine fever virus in Thailand. Proceedings of the 15<sup>th</sup> IPUS Congress, Birmingham, England, 5–9 July 1998, p. 358.

- Ruggli, N., Tratschin, J.D., Mittelholzer, C., Hofmann, M.A., 1996. Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. *J. Virol.* 70, 3478–3487.
- Sakoda, Y., Ozawa, S., Damrongwatanopokin, S., Sato, M., Ishikawa, K., Fukusho, A., 1999. Genetic heterogeneity of porcine and ruminant viruses mainly isolated in Japan. *Vet. Microbiol.* 65, 75–86.
- Simmonds, P., Mellor, J., Sakuldamrongpanich, T., Nuchaprayoon, C., Tanprasert, S., Holmes, E.C., 1996. Evolutionary analysis of hepatitis C virus found in south-east Asia: comparison with classifications based upon sequence similarity. *J. Gen. Virol.* 77, 3013–3024.
- Stadejek, T., Warg, J., Ridpath, J.F., 1996. Comparative sequence analysis of the 5'-noncoding region of classical swine fever virus strains from Europe, Asia, and America. *Arch. Virol.* 141, 771–777.
- Stadejek, T., Vilcek, S., Lowings, J.P., Ballagi-Pordany, A., Paton, D.J., Belák, S., 1997. Genetic heterogeneity of classical swine fever virus in central Europe. *Virus Res.* 52, 195–204.
- Strimmer, K., von Haeseler, A., 1996. Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Mol. Biol. E* 13, 964–969.
- Strimmer, K., von Haeseler, A., 1997. Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment. *PNAS (USA)* 94, 6815–6819.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Vanderhallen, H., Koenen, F., 1997. Molecular characterisation of classical swine fever virus isolates collected in Belgium since 1988. In Report on Annual Meeting of National Swine Fever Laboratories, Vienna, Austria, 16–17, June 1997. Published by the Commission of the European Communities, Directorate General VI/B/II.2, VI/7888/97, pp 29–32.
- Vanderhallen, H., Mittelholzer, C., Hofmann, M.A., Koenen, F. 1999 Classical swine fever virus is genetically stable in vitro and in vivo. *Arch. Virol.*, 144, 1669–1677.
- Vilcek, S., Stadejek, T., Ballagi-Pordany, A., Lowings, J.P., Paton, D.J., Belák, S., 1996. Genetic variability of classical swine fever virus. *Virus Res.* 43, 137–147.
- Vilcek, S., Stadejek, T., Takacsova, I., Strojny, L., Mojzis, M., 1997. Genetic analysis of classical swine fever virus isolates from a small geographic area. *Dtsch. Tierarztl. Wschr.* 104, 9–12.
- Vilcek, S., Belák, S., 1997. Organization and diversity of the 3'-noncoding region of classical swine fever genome. *Virus Genes* 15, 181–186.
- Vilcek, S., Paton, D.J., 1998. Application of genetic methods to study the relationship between classical swine fever virus outbreaks. *Res. Vet. Sci.* 65, 89–90.
- Vilcek, S., Paton, D., Lowings, P., Björklund, H., Nettleton, P., Belák, S., 1999. Genetic analysis of pestiviruses at the 3' end of the genome. *Virus Genes* 18, 107–114.
- Widjoatmodjo, M.N., van Gennip, H.G.P., de Smit, A.J., Moormann, R.J.M., 1999. Comparative sequence analysis of classical swine fever virus isolates from the epizootic in the Netherlands in 1997–1998. *Vet. Microbiol.* 66, 291–299.

# Application of a blocking enzyme-linked immunosorbent assay for serological monitoring of hog cholera (classical swine fever) in Poland

Z. PEJSAK \*, A. LIPOWSKI \* and M. TRUSZCZYNSKI \*\*

*Summary:* Between 1990 and 1992, serum samples from 55,478 domestic swine were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of hog cholera virus (HCV) antibodies. The amount of antibody in the sera was expressed as the mean percentage inhibition (PI). For diagnosis, the tested sera were diluted 1:2 and considered positive if the PI was less than 25%. Sera giving PI values in the range of 25-50% were retested against HCV and bovine virus diarrhoea virus (BVDV), by neutralising peroxidase-linked assay. Comparison of the serum titres obtained was used for serological diagnosis of hog cholera; the tested sera were considered negative for hog cholera if the titre for BVDV was higher than that obtained for HCV. All sera with a PI higher than 50% were considered negative for HCV and BVDV. All sera were found to be free of antibodies to HCV. BVDV antibodies were demonstrated in 0.40% of the sera tested in 1990, in 1.80% in 1991 and 1.06% in 1992.

**KEYWORDS:** Classical swine fever – ELISA – Hog cholera – Monitoring – Pestivirus – Poland.

## INTRODUCTION

Hog cholera (HC) – also known as “classical swine fever” (3) – is an infectious viral disease of domestic and wild pigs caused by a pestivirus (hog cholera virus: HCV) (4). Recently, based on new insights into the replication strategy and genome organisation of pestiviruses, these were allocated to the family Flaviviridae (earlier, pestiviruses were considered as belonging to the family Togaviridae) (7). HC remains an important disease economically, particularly in countries with a high pig population density (1). The spread of HCV strains of low and medium virulence, which cause atypical and subclinical forms of HC, underlines the significance of serological monitoring in the eradication of this disease (14).

In accordance with the OIE *International Animal Health Code* and European Community (EC) regulations (10), a country exporting swine or swine products is obliged to demonstrate freedom from HC on the basis of serological investigations.

\* National Veterinary Research Institute, Department of Swine Diseases, 57 Partyzantow Street, 24-100 Pulawy, Poland.

\*\* National Veterinary Research Institute, Department of Microbiology, 57 Partyzantow Street, 24-100 Pulawy, Poland.

In 1990, Poland implemented a system of obligatory serological screening for HCV antibodies. The primary objective of the present three-year study was to obtain further data on the seroprevalence of HC in Poland.

## MATERIALS AND METHODS

### Sera

In accordance with instructions issued by the Polish Ministry of Agriculture (Department of Veterinary Services), blood samples were collected from sows and boars kept on private farms. In 1990, tests were conducted on 17,545 field sera from all except one of the 49 provinces in Poland; in 1991, 21,378 sera from 47 provinces were tested and in 1992, 16,555 sera from 46 provinces were tested. The sera were heat-inactivated at 56°C for 30 min, and kept frozen at -20°C until testing. The quality of the serum samples varied, with regard to haemolysis and bacterial contamination.

### Serology

Blocking enzyme-linked immunosorbent assay (ELISA) (5) was used for serological screening for HCV antibody. In view of the fact that antibodies against bovine virus diarrhoea virus (BVDV) had been identified in the Polish swine population (11), the neutralisation peroxidase-linked assay (NPLA) (6, 8) was used for the differentiation of BVDV and HCV antibodies.

### Enzyme-linked immunosorbent assay

HCV antigen for coating microplates was produced using the method described by Have (5), with the modifications proposed by Dalsgaard and Overby (2). The Alfort strain of HCV was used for production of antigen in PK15 cell cultures propagated in roller bottles. Cells were scraped with a rubber policeman and pelleted by centrifugation. The cell pellet was extracted in two volumes of 20 mM tris (pH 7.2), 1% Triton X-100, 0.5 mM ethylene diamine tetra-acetic acid (EDTA) by sonication. The extract was clarified at 5,000 g for 20 min, precipitated by ammonium sulphate at 40% saturation and resuspended in 20 mM tris (pH 7.2), 0.1% Triton X-100 and 0.5 mM EDTA.

Depending on the titre, the antigen was diluted from 1:800 to 1:2,000 in carbonate buffer (pH 9.6) and placed on microplates. Plates were coated for 18 h at room temperature. After washing the plates three times with washing buffer (phosphate-buffered saline: PBS) containing 0.05% Tween 20 (PBST), test sera (diluted 1:2 in PBST) were placed in appropriate wells and incubated for 18 h at 4°C. Subsequently, microplates were washed three times with PBST. Microplates were then incubated for 1 h at 37°C with rabbit anti-HCV serum diluted 1:100-1:200. The microplates were then washed three times with PBST.

The ability of the test sera to inhibit the reaction between antigen and rabbit anti-HCV serum was examined by reaction with peroxidase-labelled anti-rabbit immunoglobulin G (IgG). Microplates were incubated with this conjugate (diluted according to the recommendations of the manufacturer) at 37°C for 30 min. After washing three times, the reaction was visualised by incubating the microplates for 15 min at room temperature in the dark with a substrate solution containing 4.6 mM ortho-phenylene diamine, 9 mM H<sub>2</sub>O<sub>2</sub> in 38 mM citric acid and sodium phosphate buffer (pH 5). After 15 min, the reaction was stopped with 0.5 N sulphuric acid. Optical densities (OD) were then recorded at 492 nm using an ELISA reader. On each microplate, two wells with negative reference sera and two wells with strong positive reference sera were included as controls.

Any uncoated sites on microplates were blocked using several blocking agents (horse serum, 1% bovine serum albumin, and 2%, 5% and 10% solutions of defatted dry bovine milk) in preliminary trials. The authors have previously shown that to be highly sensitive and specific, ELISA requires no blocking agents for the serodiagnosis of HC (12), and therefore such agents were not used.

#### Interpretation of the results

In accordance with the method described by Have (5), the antibody reactivity of the test sera was calculated as percentage inhibition (PI), where:

$$PI = \frac{(OD \text{ negative reference} - OD \text{ test serum}) \times 100}{OD \text{ negative reference} - OD \text{ strong positive reference.}}$$

In accordance with the recommendation of Have (5) and the results presented by Leforban and colleagues (9), the cut-off value for inhibition was established at 25%. Therefore, samples diluted 1:2 were considered positive if the PI was less than 25%.

(Since the completion of the present investigations, a slightly different method of calculating PI values, where:

$$PI = \frac{(OD \text{ negative reference} - OD \text{ test serum}) \times 100}{OD \text{ negative reference}}$$

has been adopted by the Standards Commission of the OIE.)

BVDV reactions may reach as high as 50% inhibition in the ELISA (5, 9). Therefore, sera giving PI values in the range of 25-50% were retested against HCV and BVDV. Testing was conducted by neutralisation assay in microplates, using a 1:25 dilution of the tested serum as the initial dilution, and results were read by NPLA (6). Results were determined by comparison of the serum titres obtained. The tested sera were considered negative for HC if the titre with BVDV was higher than that obtained with HCV. Sera with PI higher than 50% were considered HCV- and BVDV-negative.

## RESULTS

The results demonstrated that, in 1990, 71 serum samples (0.40%) had BVDV antibodies; all other samples (17,464 sera), were free from HCV and BVDV antibodies (Table I).

In 1991, 21,378 serum samples were tested. All sera were found to be free from HCV antibodies, but the prevalence of BVDV-positive sera increased to 385 (1.8%). In 1992, 16,555 serum samples were tested and again all sera were free from HCV antibodies, and 176 serum samples (1.06%) were BVDV-positive. Differences were found in BVDV seroprevalence between the different provinces. In some provinces, 5.0% of pigs tested were BVDV-seropositive, whereas in other provinces all tested samples were negative for BVDV. The comparative serology by NPLA of the sera inhibiting 25-50% of the signal usually provided a clear-cut result, showing either the absence of antibody against HCV or titres distinctly lower than the corresponding antibody titre against BVDV.

TABLE I

*Seroprevalence of antibodies to hog cholera virus (HCV) and bovine virus diarrhoea virus (BVDV) in pigs in Poland between 1990 and 1992*

Year	No. of swine tested	No. of swine with antibodies in serum			
		HCV		BVDV	
		No.	%	No.	%
1990	17,535	-	-	71	0.40
1991	21,378	-	-	385	1.80
1992	16,555	-	-	176	1.06

## DISCUSSION

The choice of cut-off value is essential with regard to the sensitivity and specificity of blocking ELISA. A high cut-off value will eliminate false-positive results, but will also decrease the sensitivity of the test, leading to a risk of obtaining false-negative results (9). The cut-off value of 25% applied in these studies has been previously determined experimentally (5, 9). Corresponding standards (positive and negative sera) prepared by the authors were used. The specificity and sensitivity of the ELISA technique used in the present study was also controlled by using a set of HCV- and BVDV-positive and HCV- and BVDV-negative sera obtained from the EC Liaison Laboratory for Classical Swine Fever in Hanover (Germany), as well as a similar set of sera supplied by the *Station de Pathologie Porcine* in Ploufragan (France). Preliminary investigations demonstrated the balance between the required sensitivity and the number of heterologous reactions which could be tolerated. Simultaneously, it was demonstrated that, because of frequent occurrence of antibodies to BVDV in swine populations, it is necessary, in addition, to use NPLA to test sera with a PI ranging from 25-50%, for antibodies against this heterologous pestivirus. This confirms the findings of a previous report (5).

Prior to 1991, approximately two million doses of hog cholera vaccine were used annually for vaccination against this disease in Poland. A programme of vaccination was followed only in State farms and in farms where garbage feed was used, while it was forbidden to vaccinate pigs in private enterprises. Therefore, according to Ministry of Agriculture regulations, only swine serum samples from private farms were tested. This excluded the possibility of obtaining false-positive reactions due to the detection of any residual vaccine.

The breeding pig population on private farms in Poland totals approximately 15 million animals. Of these, approximately 20,000 pigs are tested annually for HC seroprevalence. This represents a far greater proportion than in other European countries such as Denmark where, from a total of approximately 16 million pigs, only 4,500 are tested for HCV antibodies each year (P. Have, personal communication, 1993).

BVDV seroprevalence in Poland may be compared with that in other HCV-free countries. For example, Holm-Jensen (6) demonstrated BVDV antibodies in 6.4% of 3,000 serum samples tested in Denmark. Surveys revealed that the prevalence of naturally-occurring antibodies to BVDV in breeding pigs in Australia, Germany, the

Netherlands and Ireland ranged from 3% to 40% (6, 13). The frequent occurrence of antibodies to BVDV in swine populations, observed by other authors and in this study, justifies the need for precise determination of the cut-off value of the immunoenzymatic method applied. These findings also lend support to the use of NPLA to clarify doubtful ELISA results.

The methods used in this study may also be used for indirect (serological) differential diagnosis of infection with BVDV in pigs which have signs resembling HC (13).

### CONCLUSIONS

The blocking ELISA, applied together with NPLA, enables accurate surveillance of large numbers of samples for HCV antibodies.

All swine sera tested during the study between 1990 and 1992 were found to be free from HCV antibodies.

The study showed a non-significant increase of BVDV seroprevalence in pigs in Poland.

\*

\* \*

### APPLICATION D'UNE ÉPREUVE IMMUNO-ENZYMATIQUE DE COMPÉTITION À LA SURVEILLANCE SÉROLOGIQUE DE LA PESTE PORCINE CLASSIQUE EN POLOGNE. – Z. Pejsak, A. Lipowski et M. Truszczynski.

*Résumé* : Entre les années 1990 et 1992, des prélèvements de sérum effectués sur 55 478 porcs domestiques ont fait l'objet de recherches d'anticorps dirigés contre le virus de la peste porcine classique (hog cholera virus : HCV) à l'aide d'une épreuve enzyme-linked immunosorbent assay (ELISA). Le titrage des anticorps était exprimé en pourcentage moyen d'inhibition (mean percentage inhibition : PI). Aux fins de diagnostic, les sérums testés ont été dilués au demi et considérés comme positifs lorsque PI était inférieur à 25 %. Les sérums dont le pourcentage d'inhibition se situait entre 25 % et 50 % ont été soumis à de nouvelles recherches d'anticorps contre le virus HCV et celui de la diarrhée virale bovine (bovine virus diarrhoea virus : BVDV) à l'aide de l'épreuve peroxydasique de neutralisation. La comparaison des titres obtenus a ensuite été appliquée au diagnostic sérologique de la peste porcine : les sérums testés étaient considérés comme négatifs vis-à-vis de la peste porcine lorsque le titre du virus BVDV était supérieur à celui du virus HCV. Tous les sérums dont le pourcentage d'inhibition était supérieur à 50 % étaient considérés comme négatifs vis-à-vis des virus HCV et BVDV. Aucun anticorps du virus HCV n'a été décelé dans les sérums. Les anticorps du virus BVDV ont été mis en évidence dans 0,40 % des sérums testés en 1990, 1,80 % en 1991 et 1,06 % en 1992.

MOTS-CLÉS : ELISA – Peste porcine classique – Pestivirus – Pologne – Surveillance.

\*

\* \*

**APLICACIÓN DE UNA PRUEBA INMUNOENZIMÁTICA DE COMPETICIÓN AL MONITORAJE SEROLÓGICO DE LA PESTE PORCINA CLÁSICA EN POLONIA. – Z. Pejsak, A. Lipowski y M. Truszczynski.**

**Resumen:** Entre los años 1990 y 1992, se investigó la presencia de anticuerpos contra el virus de la peste porcina clásica en muestras de suero recogidas en 55.478 cerdos domésticos mediante una prueba inmunoenzimática (ELISA). La titulación de los anticuerpos estaba expresada en porcentaje medio de inhibición (PI). Se observaron los sueros con fines diagnósticos, diluidos en su mitad y considerados positivos cuando el PI era inferior a 25%. Los sueros cuyo PI oscilaba entre 25% y 50% fueron sometidos a nuevas búsquedas de anticuerpos contra los virus de la peste porcina clásica y de la diarrea viral bovina, por medio de la prueba peroxidásica de neutralización. La comparación de los títulos obtenidos se aplicó a continuación para establecer el diagnóstico serológico de la peste porcina clásica: los sueros investigados se consideraron negativos en relación con la peste porcina clásica cuando el título del virus de la diarrea viral bovina era superior al obtenido del virus de la peste porcina clásica. Todos los sueros cuyo PI era superior a 50% se consideraron negativos en relación con los virus de la peste porcina clásica y de la diarrea viral bovina. No se identificó ningún anticuerpo del virus de la peste porcina clásica en los sueros. Los anticuerpos del virus de la diarrea viral bovina se manifestaron en 0,40% de los sueros investigados en 1990, en 1,80% de los investigados en 1991 y en 1,06% de los investigados en 1992.

**PALABRAS CLAVE:** Control – ELISA – Peste porcina clásica – Pestivirus – Polonia.

\*  
\* \*

**REFERENCES**

1. DAHLE J. & LIESS B. (1992). – A review on classical swine fever infections in pigs: epizootiology, clinical disease and pathology. *Comp. Immun. Microbiol. infect. Dis.*, **15**, 203-211.
2. DALSGAARD K. & OVERBY E. (1976). – Vaccination against hog cholera (classical swine fever) with a detergent split vaccine. *Acta vet. scand.*, **17**, 465-474.
3. FAO/OIE/WHO (1991). – Animal Health Yearbook. FAO Animal Production and Health Series No. 31.
4. FENNER R. (1976). – Classification and nomenclature of viruses. *Intervirology*, **7**, 1-116.
5. HAVE P. (1984). – Detection of antibodies against swine fever virus by enzyme-linked immunosorbent assay (ELISA). *Acta vet. scand.*, **25**, 462-465.
6. HOLM-JENSEN M. (1985). – Screening for neutralizing antibodies against hog cholera- and/or bovine viral diarrhoea virus in Danish pigs. *Acta vet. scand.*, **26**, 72-80.
7. HORZINEK M.C. (1991). – Pestivirus taxonomic perspectives. *Arch. Virol.*, (Suppl. 3), 1-5.
8. HYERA J.M., LIESS B. & FREY H.R. (1987). – A direct neutralizing peroxidase-linked antibody assay for detection and titration of antibodies to bovine viral diarrhoea virus. *J. vet. Med., B.*, **34**, 227-239.

9. LEFORBAN Y., EDWARDS S., IBATA G. & VANNIER P. (1990). – A blocking ELISA to differentiate hog cholera virus antibodies in pig sera from those due to other pestiviruses. *Ann. Rech. vét.*, **21**, 120-129.
  10. LIESS B. (1988). – Serology. *In* Classical swine fever and related viral infections. Martinus Nijhoff Publishers, Boston, Dordrecht & Lancaster, 115-142.
  11. PEJSAK Z. & KOLACZ J. (1991). – Application of ELISA for detection of antibody against swine fever in screening studies. *Medycyna wet.*, **47**, 400-401.
  12. PEJSAK Z. & LIPOWSKI A. (1992). – Evaluation of different blocking agents for hog cholera serodiagnosis. *In* Proc. International Pig Veterinary Society Congress, The Hague, 17-20 August, 146.
  13. TERPSTRA C. & WENSVOORT G. (1988). – Natural infections of pigs with bovine viral diarrhoea virus associated with signs resembling swine fever. *Res. vet. Sci.*, **45**, 137-142.
  14. VAN OIRSCHOT J.T. (1988). – Description of the virus infection. *In* Classical swine fever and related viral infections (B. Liess, ed.). Martinus Nijhoff Publishers, Boston, Dordrecht & Lancaster, 1-25.
-