Veterinaria Italiana, **44** (2), 305-318

Genetic variation of classical swine fever virus based on palindromic nucleotide substitutions, a genetic marker in the 5′ untranslated region of RNA

Massimo Giangaspero & Ryô Harasawa

Summary

Forty-three strains of classical swine fever (hog cholera) virus (CSFV) from outbreaks in pigs in Europe, Asia and America, two strains from commercial CSFV modified live vaccines and a strain isolated from a diseased lamb from Spain were subjected to analyses of nucleotide sequence variations in the 5′ terminal region of the genome. These isolates were divided into three clusters, namely: CSFV-1, CSFV-2, and CSFV-3, based on palindromic nucleotide substitutions in the 5′ untranslated region (UTR). The homology degree, according to nucleotide base pairing variation in the secondary palindromic structure of the three variable loci V1, V2 and V3, was 60% in the CSFV species, with a mean divergence value of 6.19 base pairs (bp). relatedness within genotypes ranged from 71.11% to 100%, with mean divergence values from 5.5 to 0.73 base pairs. Subgenotypes showed a divergence ranging from 1 to 9 base pairs within the genotype. Genotype CSFV-1 revealed 15 base pair combinations with 13 divergent base pairs, resulting in 4 subgenotypes with 6 variants in subgenotype CSFV-1.1, including the reference strain Brescia and 6 variants in subgenotype CSFV-1.2, including the Alfort reference strain. Subgenotypes CSFV-1.3 and CSFV-1.4 comprised one and two variants, respectively. Genotype CSFV-2 was represented by the Spanish ovine isolate 5440/99 and the genotype CSFV-3 included the Japanese strains Okinawa/86 and Kanagawa/74. CSFV genotypes revealed a strong relationship with Border disease virus strains, showing relatively low divergence values when compared to other pestivirus species. Evaluation of nucleotide base pair divergence among genotypes and expression of evolutionary changes in the CSFV species led to the construction of a phylogenetic tree based on secondary structure.

Keywords

Classical swine fever, Genotypes, Hog cholera, Nucleotide, Palindromic nucleotide substitution, Pestivirus.

Variazione genetica della specie del virus della peste suina classica basata sulle sostituzioni palindromiche nucleotidiche, un marker genetico nella regione non tradotta 5′ dell'RNA

Riassunto

Quarantatre ceppi di virus della peste suina classica (PSC), provenienti da focolai in suini dell'Europa, Asia e America, due ceppi di vaccini vivi modificati commerciali di PSC e un ceppo isolato da un agnello malato provenienti dalla Spagna sono stati sottoposti ad analisi delle variazioni delle sequenze nucleotidiche a livello della regione terminale 5′ del genoma. Questi isolati sono stati suddivisi in tre gruppi, PSC-1, PSC-2 e PSC-3, sulla base delle sostituzioni palindromiche nucleotidiche nella regione non tradotta 5′ (UTR). Il grado di omologia, secondo la variazione delle paia di basi

Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan
giangasp@iwate-u.ac.jp

nucleotidiche nella struttura palindromica secondaria dei tre loci variabili V1, V2 e V3, era 60% nella specie PSC, con un valore medio di divergenza di 6,19 paia di basi. La relazione nei genotipi variava da 71,11% a 100%, con un valore medio di divergenza da 5,5 a 0.73 paia di basi. I sotto-genotipi hanno mostrato una divergenza variante da 1 a 9 paia di basi nel genotipo. Il genotipo PSC-1 ha rivelato 15 combinazioni di paia di basi con 13 paia di basi divergenti, risultando in 4 sotto-genotipi con 6 varianti nel sotto-genotipo PSC-1.1, includendo il ceppo di referenza Brescia, e 6 varianti nel sotto-genotipo PSC-1.2, includendo il ceppo di referenza Alfort. I sotto-genotipi PSC-1.3 e PSC-1.4 comprendevano rispettivamente una e due varianti. Il genotipo PSC-2 era rappresentato dall'isolato ovino Spagnolo 5440/99, e il genotipo PSC-3 includeva i ceppi Giapponesi Okinawa/86 e Kanagawa/74. I genotipi di PSC hanno mostrato una relazione significativa con i ceppi del virus della Border disease*, con valori relativamente bassi di divergenza se comparati alle altre specie di pestivirus. La valuazione della divergenza delle paia di basi nucleotidiche tra genotipi, espressione dei cambi evolutivi nella specie PSC, ha permesso la costruzione di un albero filogenetico basato sulla struttura secondaria.*

Parole chiave
Genotipi, Nucleotide, Peste suina classica, Pestivirus, Sostituzioni palindromiche nucleotidiche.

Introduction

Classical swine fever (CSF) virus (CSFV), a species of the genus *Pestivirus* in the family *Flaviviridae* along with bovine viral diarrhea virus-1 (BVDV-1), bovine viral diarrhea virus-2 (BVDV-2), Border disease virus (BDV) and a tentative 'giraffe' species (4), is the causative agent of classical swine fever or hog cholera, a cosmopolitan disease that affects pigs and which has a relevant impact on animal production. The disease is subjected to statutory controls that involve the slaughter of affected pigs and the placing of restrictions on movements of pigs from affected areas. Despite intensive eradication efforts, CSF has recurred in many parts of the world. Wild boar have been suspected to be an important reservoir of CSFV (20).

The CSFV genome, a single-stranded, positive polarity RNA, is made up of a sequence of about 12 500 nucleotides, with a relatively long 5′-untranslated region (UTR) upstream of the polyprotein open-reading frame (3). The 5′-UTR nucleotide sequence is highly conserved among all members within the genus *Pestivirus*, thus being useful for the characterisation of species or genotypes. Although well conserved, the 5′-UTR has been known to contain at least three variable loci. The nucleotide substitutions at these variable loci are particularly important because the 5′-UTR of positive-sense RNA viruses generally include regulatory motifs which are indispensable to viral survival. The secondary structure of the 5′-UTR can be divided into four domains, A-D, with domain D encompassing the two-thirds in the 3′ region of the 5′-UTR predicted to fold into a complex palindromic stem-loop structure (3, 11), critical region of the 5′-UTR, containing an internal ribosomal entry site, responsible for translational, transcriptional and replicational events in pestiviruses (3). Therefore, point mutations at the 5′-UTR which occur continuously and at random throughout the virus genome at every replication phase at a rate of 10^{-3}~10^{-4} per incorporated nucleotide in RNA viruses, have a high probability of incompatibility with viral survival. Thus, stable nucleotide variations at this level assume importance in terms of virus evolutionary history. Nucleotide sequences at the three variable loci, V1, V2 and V3, in the 5′-UTR of pestiviruses have been shown to be palindromic and are capable of forming a stable stem-loop structure peculiar to each *Pestivirus* species. Nucleotide substitutions in the stem regions always occur to maintain a palindromic sequence and thereby form a stable stem-loop structure and this type of mutation was referred to palindromic nucleotide substitutions (PNS) (13). The observation of nucleotide variations among virus strains at the level of the three specific palindromes in the 5′-UTR has been conceived as a simple and practical procedure for

genotyping (13). The method named PNS proposed keys of identification at the genus, species and genotype levels consisting of three aspects, as follows:

- palindromic sequences: three strictly conserved regions representing palindromic structures identified in the 5′-UTR of the same genotype
- variable loci: three variable loci, V1, V2 and V3 identified in the 5′-UTR, which correspond to the three palindromic structures; these variations are characteristic and well conserved, either among all the different species or specifically within a single species
- nucleotide sequences: in the nucleotide sequence of each variable locus, three kinds of base pairings can be classified. They are:
  a) inter-species specific base pairing in the stem regions
  b) species specific base pairing in the stem regions
  (c) highly variable and uncharacteristic base pairing.

According to primary structure analysis, by sequence alignment and construction of phylogenetic trees, the most common method for the classification of the *Pestivirus* isolates, strains of CSFV have been divided into two genomic groups, I and II, based on phylogenetic analyses of the 5′-UTR, 3′-UTR, E2 (gp55) gene, or NS5B (putative RNA-dependent RNA polymerase) gene (21, 35, 36). Group I includes the reference strain Brescia, together with old American, European and Japanese isolates, and group II includes the Alfort reference strain and recent European isolates. Secondary structure analyses segregated into group I and group II strains among Japanese isolates by sequence analysis of the 5′-UTR and the 3′ end of the viral genome, including a partial region of the NS5B gene and the 3′-UTR (14). The Japanese strain Kanagawa/74 has been considered to be a disparate strain among CSFV strains according to phylogenetic analyses (14, 21, 35) and classified with strain Okinawa/86 in a distinct CSFV genotype (14).

In the present study, classification of CSFV species according to PNS genotyping method was established. To determine genotypic variations in the species, the 5′-UTR genomic region of forty-three CSFV strains, including previously evaluated strain sequences, were analysed using the PNS procedure, applying qualitative and quantitative evaluation of nucleotide variations.

Materials and methods

The nucleotide sequences in the 5′-UTR of forty-three CSFV strains were obtained from the DNA databases and from publications or requested directly from authors, when not deposited in databases. Strains of the tested nucleotide sequences are presented in Table I. The majority of the strains tested, including reference strains, originated from outbreaks in pigs from Europe, Asia and America. Strains GPE(–) and KC were recovered from CSFV modified live vaccines (Matsuoka-Kagaku Co., Ltd., Tokyo) (9). Strain 5440/99 was isolated from a diseased lamb from Spain (18).

The nucleotide sequences of relevant regions were obtained from the data published on *Pestivirus* reference strains, Alfort (22), Brescia (24) and Osloss (2). Nucleotide sequences of the other pestivirus strains obtained from DNA databases were as follows (accession number is given in brackets): strains NADL (M31182), Singer (L32875) and Oregon (L32876) for BVDV-1a; strains Draper (L32880) and NY-1 (L32879) for BVDV-1b; strains PT810 (Z79766), SE5572 (Z79770) and Europa (AB000898) for BVDV-1c; strains EBTr (D50817), 890 (L32886) and CD87 (L32887) for BVDV-2; strains BD31 (U70263), Moredun (U65023) and Ch1Es (D50816) for BDV.

Nucleotide sequences were aligned using the method proposed by Higgins *et al*. (16) using the DNASIS program package (Hitachi Software Engineering, Co., Yokohama). The phylogenetic trees based on the 5′-UTR were constructed using the unweighed pair-group method with arithmetic averages (UPGMA) described by Sneath and Sokal (30). Nucleotide sequence secondary structures were predicted according to the algorithm of Zuker and Stiegler (38). The minimum free energy was calculated using the method of Freier *et al*. (6).

Table I
Palindromic nucleotide substitution method for genetic evaluation
Classical swine fever virus strains classified according to the PNS method at the 5′ untranslated region of RNA

Genotype	Strain	Origin	Country	Accession No.	Reference
CSFV-1.1	39	Pig	China	AF407339	Wu *et al*., unpublished
CSFV-1.1	Alfort 187	Pig	France	X87939	28
CSFV-1.1	Alfort A19	Pig	France	U90951	Smondack *et al*., unpublished
CSFV-1.1	Brescia	Pig	Italy	M31768	24
CSFV-1.1	C strain	Pig	China	Z46258	25
CSFV-1.1	CAP	Pig	Switzerland	X96550	Tratschin *et al*., unpublished
CSFV-1.1	cF114	Pig	China	AF333000	Mingxiao *et al*., unpublished
CSFV-1.1	Eystrup	Pig	Germany	AF326963	23
CSFV-1.1	GPE (–)	Vaccine	Japan	AB019152	14
CSFV-1.1	HCLV	Pig	China	AF091507	Wang *et al*., unpublished
CSFV-1.1	Hokkaido/66	Pig	Japan	AB019154	14
CSFV-1.1	Ibaraki/66	Pig	Japan	AB019156	14
CSFV-1.1	Ibaraki/81-115	Pig	Japan	AB019158	14
CSFV-1.1	Ibaraki/81-20	Pig	Japan	AB019160	14
CSFV-1.1	Ibaraki/81-38	Pig	Japan	AB019162	14
CSFV-1.1	Ibaraki/81-40	Pig	Japan	AB019164	14
CSFV-1.1	KC	Vaccine	Russia	AF099102	9
CSFV-1.1	LOM	Pig	Japan	AB019655	29
CSFV-1.1	Miyazaki/81	Pig	Japan	AB019168	14
CSFV-1.1	Nakamura/66	Pig	Japan	AB019170	14
CSFV-1.1	Shimen	Pig	China	AF092448	Huang *et al*., unpublished
CSFV-1.1	Vac A	Pig	United States	L42435	31
CSFV-1.1	Yamanashi/69	Pig	Japan	AB019182	14
CSFV-1.2	17-93	Pig	Poland	L42413	31
CSFV-1.2	Alfort	Pig	France	J04358	22
CSFV-1.2	Chiba-80	Pig	Japan	AB019659	29
CSFV-1.2	Osaka/51	Pig	Japan	AB019174	14
CSFV-1.2	Osaka/71	Pig	Japan	AB019176	14
CSFV-1.2	Pader	Pig	Germany	AY072924	34
CSFV-1.2	Shizuoka/73	Pig	Japan	AB019180	14
CSFV-1.2	Switzerland 1/93	Pig	Switzerland	AF045068	17
CSFV-1.2	Switzerland 2/93′	Pig	Switzerland	AF045069	17
CSFV-1.2	Switzerland 3/93/1′	Pig	Switzerland	AF045070	17
CSFV-1.2	Switzerland 3/93/2′	Pig	Switzerland	AF045071	17
CSFV-1.2	Switzerland 4/93′	Pig	Switzerland	AF045072	17
CSFV-1.2	Venhorst	Pig	Netherlands	AF084049	37
CSFV-1.2	VRI4762	Pig	Malaysia	L42437	31
CSFV-1.3	Saitama/81	Pig	Japan	AB019178	14
CSFV-1.4	Fukuoka/72	Pig	Japan	AB019150	14
CSFV-1.4	Honduras	Pig	Honduras	L42426	31
CSFV-2	5440/99	Sheep	Spain	AY159514	18
CSFV-3	Okinawa/86	Pig	Japan	AB019172	14
CSFV-3	Kanagawa/74	Pig	Japan	AB019166	14

CSFV classical swine fever virus

Three variable regions, V1, V2 and V3, at the 5′-UTR were used for genotyping based on palindromic nucleotide substitutions (13) and compared with those of other pestiviruses BVDV-1, BVDV-2, BDV and the 'giraffe' strain.

Base pair (bp) variations shared in the genus and at the level of the CSFV species were identified as characteristic PNS. Within the CSFV species, genotypes were identified based on characteristic nucleotide base pairings.

Homology degree was determined in the genus *Pestivirus* and among species and genotypes according to base pairing nucleotide variation in the secondary palindromic structure of the three variable loci (V1, V2 and V3). relatedness among genotypes within the CSFV species, and segregation of genotypes in subgenotypes and variants was evaluated according to changes in nucleotide base pairs at the level of stems and loops of the three (V1, V2 and V3) palindromic variable loci. Among genotypes, homology was evaluated in terms of shared base pairs out of the total of 45 palindromic positions (23 in V1, 12 in V2 and 10 in V3), identifying a mean divergence value. Qualitative and quantitative elements were considered for genotype and subgenotype determination. The divergence limit value for genotype determination corresponded to 9 base pair variation, proportionally related to species determination value of 13, equivalent to 35% of variation out of the total of considered positions at genus level. Subgenotypes were identified according to a divergence that did not exceed 6 base pairs in the group, including single variants, expression of isolate sequence changes. The G-C change in G*U base pairing, transitional substitution with low importance in terms of genotype characteristics, was considered an exception in the genotype. Loop sequences were also considered due to the relatively low variability compared to other pestivirus species.

A phylogenetic tree was constructed on the basis of the secondary structure through the evaluation of nucleotide base pair divergence among genotypes and expression of evolutionary changes in the CSFV species.

Results

The forty-three strains of the CSFV species of different geographical origin were classified according to the PNS genotyping method at the 5′-UTR of the viral RNA. The observation made on the nucleotide sequences of the three variable loci at the level of the 5′-UTR genomic region of *Pestivirus* strains enabled the identification of consensus motifs shared by all the *Pestivirus* species. The characteristic PNS were identified at genus, species and genotype levels (Table II). The base pairings were defined by numbering from the bottom of each variable locus. Eleven PNS were characteristic to the genus. In V1, with 21 base pairs of size, the C_C bulge was in position 11, A-U in position 10, C-G in position 8, U-A in position 7, A in position 6, U*G in position 5, U in position right nucleotide and G-C in position 4. In V2: GGGGU loop sequence, C-G was in position 8 and C-G in position 3.

The CSFV shared a species-characteristic PNS U-A pairing in position 13, with an exception made for strains Kanagawa/74 and Honduras with U*G, common to the V1 locus, a U-A pairing, common to the V3 locus in position 2, and U or C in position 8 (with an exception made for strains Saitama and 5440/99: A) at the top of the V3 loop.

The evaluation of changes in nucleotide base pairs at the palindromic structure level and the expression of relatedness and divergence among CSFV genotypes, revealed eleven prevalent positions showing base pairs that represented over 80% of the strain sequences in the species. Five prevalent positions were located in V1 at positions 9, 13, 14, 18 and 20; five were located at V2 locus in positions 1, 2, 5, 7 and 9. In V3 locus, position 1 was prevalent in the species. Low variable positions (LVP), showing base pairs representing less than 80% within the species and located in the stem sequences, were identified for genotyping and subgenotyping determination: LVP type 2 (two variants) in V2/6; type 3 (three variants) in V1/15, and type 4 (four variants) in V3/6. LVP located in the loop structures were identified at the V1 level in position 19 and in V3 in positions 7

Table II

Palindromic nucleotide substitutions characteristic to genotypes of classical swine fever virus species of the genus *Pestivirus*

The position of base pairings is defined by numbering from the bottom of the variable locus

Genotype/subtype	Variable locus	Palindromic nucleotide substitution (PNS) characteristics
Genus characteristic PNS markers		
	V1	Absence in position 22; size of V1 generally not exceeding 21 base pair (exception U)
		C_C bulge in position 11
		A-U in position 10
		C-G in position 8 (exceptions U*G, U-A and G_G bulge)
		U-A in position 7 (exception A_A bulge)
		A in position 6 (exception G)
		U*G in position 5
		U in position 5 right nucleotide
		G-C in position 4
	V2	GGGGU loop (exception GGGGC)
		C-G in position 8 (exception U*G)
		C-G in position 3 (exception U*G or C_U bulge)
Classical swine fever virus species characteristic PNS markers		
	V1	U-A in position 13 (exception made for strains Honduras and Kanagawa/74: U*G)
	V3	U-A in position 2
		U or C in position 8 (exception made for strains Saitama and 5440/99: A)
Classical swine fever virus genotypes characteristic PNS markers		
CSFV-1	V1	A_C bulge in position 15 (exception made for strains 17-93, Fukuoka/72 and Honduras: G-C)
	V2	U-A in position 5
		G:Y in position 7
	V3	A-U in position 1
CSFV-1.1	V2	A-U in position 1
		A-U in position 6
	V3	C in position 8 (exception made for strain Ibaraki/81-115: U)
CSFV-1.2	V2	A-U in position 1
		G-C or G*U in position 6
	V3	U in position 8 (exception made for strain Pader: C)
CSFV-1.3	V2	A_C bulge in position 1
		G*U in position 6
	V3	A in position 8
CSFV-1.4	V1	G-C in position 15
	V2	A-U in position 1
		A-U in position 6
	V3	C in position 8
CSFV-2	V1	G-C in position 15
		A_G bulge in position 19
		U-A in position 20
	V2	A-U in position 5
		A_C bulge in position 7
	V3	A-U in position 1
		U_C bulge in position 6
CSFV-3	V1	A-U in position 15
	V2	U-A in position 5
		G-C in position 7
	V3	A_G bulge in position 1

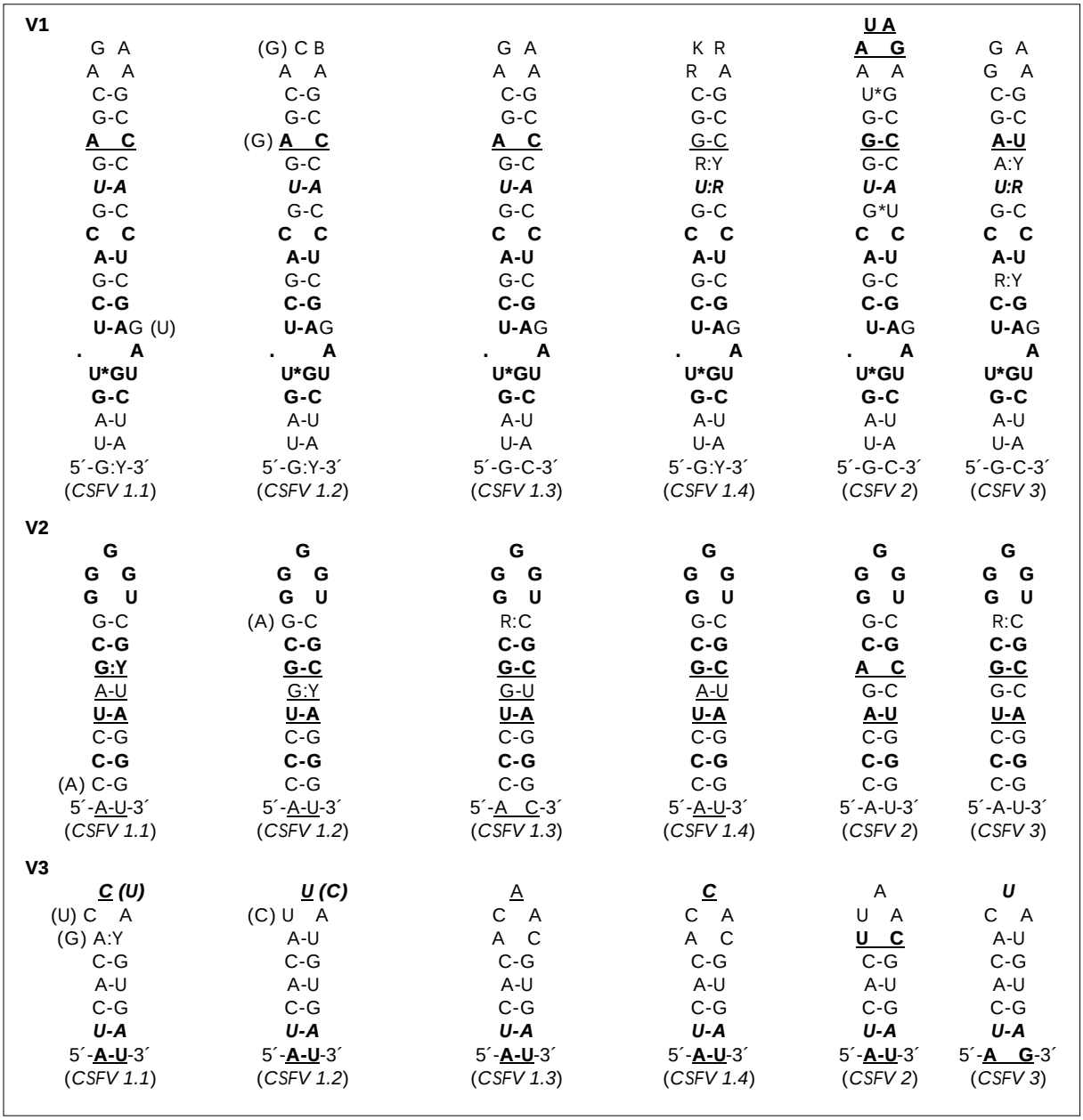
and 8. Among prevalent positions, six were represented in more than 94% of the strains observed, two in 88% and three in 82%. Divergent base pairs did not generally exceed 5.89%. Among LVPs, the G-C or G*U base pair in V2 in position 6 represented 58.82%. The position was shared with an A-U base pairing representing the 41.18% of the CSFV strains. In V1 locus, A_C bulge represented 64.70% at position 15 shared with G-C (23.52%) and A-U (11.76%). In V3 at position 6, A-U base pairing was the most common among strains with 64.70%. A_C bulge represented 23.52% and G-C or U C pairings represented only 5.89%.

Within the CSFV species, based on characteristic nucleotide base pairings, three genotypes were identified, namely: CSFV-1, CSFV-2 and CSFV-3. Figure 1 shows the V1, V2 and V3 palindromic loci in the 5′-UTR of the CSFV species. The base pairings characteristic to genus (PNS genus-specific) and those characteristic to the species CSFV (PNS species-specific) are represented within the three genotypes. Out of 43 tested strains, 40 strains belonged to the genotype CFSV-1. Strains Brescia, Ibaraki/66, Ibaraki/81-20, Ibaraki/81-38, Ibaraki/81-40, Ibaraki/81-115, 39, cf114, Alfort A19, Alfort 187, CAP, KC, Shimen, C strain, Eystrup, GPE (–), Hokkaido/66, LOM, Miyazaki/81, Vac A, Yamanashi/69, Nakamura/66 and HCLV belonged to the subgenotype CFSV-1.1, defined type Brescia, with specific PNS at V2, A-U in position 1; A-U in position 6 and V3 C in position 8 (with an exception made for strain Ibaraki/81-115: U). Fourteen strains, namely: Alfort, Switzerland 1/93, Switzerland 2/93, Switzerland 3/93/1, Switzerland 3/93/2, Switzerland 4/93, Venhorst, VR14762, Shizuoka/73, Osaka/71, Osaka/51, Pader, Chiba-80 and 17-93 belonged to the CSFV-1.2, defined type Alfort, with the characteristic base pairings identified in V2, A-U in position 1, G-C or G*U in position 6 and in V3 U in position 8 (with an exception made for strain Pader: C). One strain, Saitama/81, belonged to CSFV-1.3 with A_C bulge in V2 in position 1 and A in V3 in position 8. Strains Fukuoka/72 and Honduras were clustered to the subgenotype CFSV-1 4, showing in the V1 locus G-C in position 15, while, with the exception of strain 17-93, all other starins belonging to genotype CSFV-1 showed A_C bulge.

One ovine strain, 5440/99, was assigned to the genotype CSFV-2, characterised by A-U in position 5 and A_C bulge in position 7 in V2 and U_C bulge in position 6 in V3. Two strains, Okinawa/86 and Kanagawa/74, showed higher divergence in the species and were assigned to genotype CSFV-3, defined type Okinawa. Characteristic base pairings were identified in the V1 and V3 loci. A Watson-Crick base pairing of A-U at position 15 from the bottom of a stem-loop structure for the V1 region, which was different from the other types of CSFV, was same as that of some BDV strains. Furthermore, a mismatched pairing by A and G at the bottom of a stem-loop structure was characteristic of the V3 region.

The strains are listed in Table I, including the reference of the depositor of the sequence in the DNA database. The strains allocated in genotypes CSFV-1 showed a cosmopolitan distribution for Europe, Asia and America. Genotype CSFV-2 represented a specific cluster in the species with a uncommon ovine origin. Genotype CSFV-3 appeared to be limited to Japan. Nucleotide sequence alignment and a phylogenetic tree constructed from the 5′-UTR of the CSFV strains along with other pestiviruses by using the UPGMA gave similar results (data not shown).

The degree of homology, according to base pairing nucleotide variation in the secondary palindromic structure of the three variable loci V1, V2 and V3, was 60% in the CSFV species (27 highly conserved positions out of 45 considered), with a mean divergence value of 6.19 base pairs. Relatedness within genotypes ranged from 71.11% to 100% (CSFV-1: 71.11%; CSFV-2: 100%; CSFV-3: 91.11%), with mean divergence values ranging from 0.73 to 5.5 base pairs from prevalent secondary structure sequences in the species (CSFV-1: 0.73; CSFV-1.1: 0.33; CSFV-1.2: 0.33; CSFV-1.3: 1; CSFV-1.4: 3; CSFV-2: 4; CSFV-3: 5.5).



– Watson-Crick base pairings
* tolerated pairings in secondary structure
: interchangeable base pairings
M= A or C; R= A or G; W= A or U; S= C or G; Y= C or U; K= G or U; Z= A or C or G; H= A or C or U; D= A or G or U; B= C or G or U; N= A or C or G or U

Figure 1
V1, V2 and V3 palindromic loci in the 5′- untranslated region of the *Pestivirus* classical swine fever virus species
Base pairings characteristic to the genus (palindromic nucleotide substitutions genus-specific) are shown in bold
The characteristic base pairings of the classical swine fever virus species (palindromic nucleotide substitutions species-specific) are presented in bold and in italics

In genotype CSFV-1 base pair divergence among subgenotypes ranged from 1 to 9, in genotype CSFV-3 divergence was 4 base pairs. Genotype CSFV-1 revealed 15 base pair combinations with 13 divergent base pairs, resulting in 4 subgenotypes allocated according to their divergence in the genotype and the divergence in the species with 6 variants in subgenotype CSFV-1.1, including the Brescia reference strain and 6 variants in subgenotype CSFV-1.2, including the Alfort reference strain. Subgenotypes CSFV-1.3 and

CSFV-1.4 comprised one and two variants, respectively. Genotype CSFV-3 included two variants represented by the Japanese strains Okinawa/86 and Kanagawa/74.

Divergence values among CSFV genotypes, according to changes in nucleotide base pairs (Table III), through their mean values, provided clear indications on relatedness among CSFV genotypes and divergence in the species, showing values of divergence ranging from 0.73 for genotype CSFV-1 to 5.5 for genotype CSFV-3. At the genotype level, values below 9 expressed the relatively low heterogeneity among strains in genotypes. Homology among genotypes ranged from low values indicating strong correlation with divergence less then base pairs mean value 9, as 8.46 and 7.97 between genotype CSFV-1 and CSFV-2 and CSFV-3, respectively. Higher values (up to 13), indicated a range of divergence among genotypes reaching marked genetic distance, for example between genotype CSFV-2 and CSFV-3.

Table III
Relationships between classical swine fever virus genotypes according to changes in nucleotide base pairs
Mean values of base pair divergence among genotypes

Genotype	MV	CSFV-1	CSFV-2	CSFV-3
CSFV-1	0.73	4.63		
CSFV-2	4	8.46	0	
CSFV-3	5.5	7.97	12.5	4

MV mean values of base pair divergence in the species

Comparison of CSFV strains with other pestivirus species provided a clear differenciation from the BVDV-1, BVDV-2 and BDV species and from Pronghorn and giraffe pestivirus strains. CSFV showed a strong relationship with BDV in terms of the very low values of divergent base pairs in the palindromic structures, with a mean divergence value of 13.56, when compared to divergence reported between other pestivirus species (Fig. 2). However, the correct segregation was evident at the level of conserved CSFV characteristic PNS at the V1 and V3 loci levels.

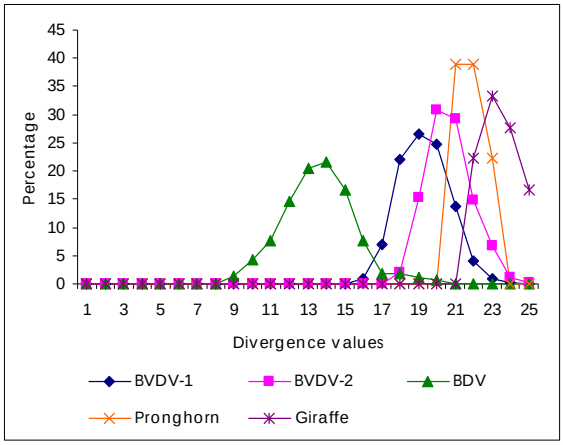
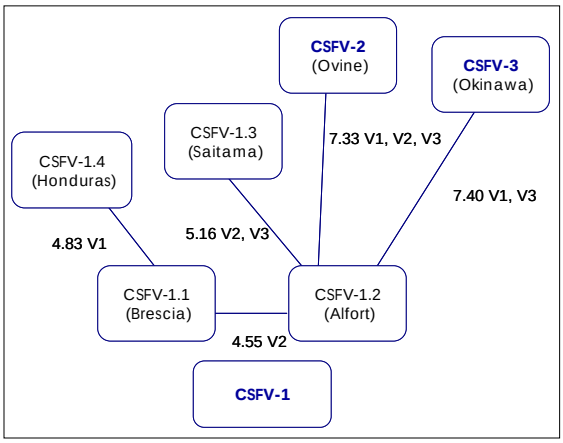


Figure 2
Comparison of classical swine fever virus strains with other pestivirus species according to changes in nucleotide base pairs

Evaluation of nucleotide base pair divergence among genotypes and expression of evolutionary changes in the CSFV species enabled the construction of a secondary structure based phylogenetic tree. The phylogenetic tree presented in Figure 3 was constructed according to the mean values of base pair divergence and indicated the correlations between genotypes identifying two main branches in the species related to subgenotypes CSFV-1.1, type Brescia, and CSFV-1.2, type Alfort. The location of base pair changes in the three variable loci was identified for each evolutionary step generating the different genotypes. The relatedness among genotypes was characterised by shared base pairs and the occurrence of further stable mutations, confirming the rationale of the PNS method and the reliability of the genotype identification keys. Mean values of divergent base pairs, indicated and quantified the genetic relatedness between genotypes in the species. These values reflected nucleotide characteristics in the specific base pairs which were either shared by related genotypes or were divergent, indicating the genetic distance between them. This aspect was fundamental, since characteristic and not shared base pairs were very limited and only partially allowed the discrimination of genotypes in the species, providing only qualitative indications of segregated genotypes that were not sufficiently

explanatory of the genetic relatedness. To define a rationale for identification, genotypes were also identified by a specific combination of base pairings in the sequence. These base pairings were non-specific when considered separately and expressed as phylogenetic markers.



CSFV classical swine fever virus

Figure 3
Schematic phylogenetic tree based on nucleotide changes in the secondary structure of classical swine fever virus genotypes
Mean values of base pair divergence and variable locus location indicate evolutionary changes

Within genotype CSFV-1, subgenotype CSFV-1.1, type Brescia, was identified by a specific combination of three base pairings (A-U in positions 1 and 6 of V2, and mainly C in position 8 in V3). Subgenotype CSFV-1.2, type Alfort, was characterised by A-U in position 1 of V2, G-C or G*U in position 6 in V2, and mainly U in V3, revealing a specific mutation at these levels that was divergent from CSFV-1.1. The subgenotype CSFV-1.1 presented a common genetic structure with subgenotype CSFV-1.4, sharing specific PNS A-U in positions 1 and 6 of V2 and C in position 8 of V3. However, in CSFV-1.4, divergence was evident at the level of V1 locus in position 15 with specific mutation with G-C base pairing, shared only with the ovine strain. Subgenotype CSFV-1.2 presented a common genetic structure with subgenotype CSFV-1.3, as well as with genotypes CSFV-2 and CSFV-3, suggesting their hypothetical origin. In subgenotype CSFV-1.3, type Saitama, the characteristic G-C or G*U base pair of CSFV-1.2 in V2 was conserved with the exception of a stable mutation that occurred at the V2 level, changing the characteristic A-U in position 1 in a A_C bulge and A in position 8 of V3 generating the new genotype. Genotype CSFV-2, ovine type, showed an unchanged characteristic base pair of CSFV-1.2 in V2 in position 6 and a stable mutation occurred at the level of V2 and V3 A-U in position 5 and A_C bulge in position 7, and U_C bulge in position 6 and A in position 8, respectively, generating the new genotype. Specific stable mutations suggested the generation of genotype CSFV-3, type Okinawa, the characteristic base pairs of CSFV-1.2 in V2 remained conserved, in V3 the A-U base pair in position 1 changed with an A_G bulge. In the V1 locus, a stable mutation appeared at the level of position 15 with A-U.

Discussion

The observation made on the nucleotide sequences of the three variable loci at the level of the 5′-UTR genomic region of CSFV strains, according to the PNS genotyping method, led to the identification of consensus motifs shared by all the *Pestivirus* species. The characteristic PNS were identified at genus, species and genotype levels (Table I). Genus-specific base pairings were positioned in the V1 and V2 loci. The CSFV strains could be clearly differentiated from the BVDV-1, BVDV-2 and BDV species and from Pronghorn and giraffe pestivirus strains. The CSFV shared characteristic base pairs that were common to the V1 and V3 loci. Three genotypes were classified within the species leading to the clear allocation of the 43 strains of varying geographical origins. Table I lists the strains and includes the reference of the depositor of the sequence in the DNA database. Pig isolates from genotype CSFV-1 represented the principal group within the species with 40 strains, showing a cosmopolitan distribution in North and Central America, Asia and Europe. CSFV-1.3 and CSFV-3 strains were limited to Japan and the isolate from genotype CSFV-2 was from Europe.

The determination of subgenotypes and subgenotype variants of CSFV genotypes, according to divergent nucleotide base pairs at the level of palindromic variable loci, showed that strains belonging to genotypes CSFV-1.1 and CSFV-3 were the most heterogenic in the species. The other genotypes showed higher homogeneity. Heterogeneity in the species was due also to the affinity demonstrated by strains such as Kanagawa/74 or Fukuoka/72 and Honduras for the BDV species. The relatedness with BDV was evident in terms of the very low values of divergent base pairs in the palindromic structures, when compared to divergence reported between other pestivirus species. However, a clear discrimination was obvious at the level of conserved CSFV characteristic PNS at the level of the V1 and V3 loci.

Clinical isolates of CSFV from outbreaks of CSF in Japan were divided into two groups, H and B, on the basis of differences in pathogenicity by experimental infections as well as in antigenicity of serum neutralisation titres using polyclonal anti-BVDV immune sera prepared in pigs. Group H (CSFV that causes a typical form of the disease) comprised CSFV strains Hokkaido/66, Ibaraki/66, Yamanashi/69 and Fukuoka/72 that did not react with anti-BVDV sera and group B (BVDV-related CSFV that causes a chronic form of disease) comprised strains Osaka/71, Shizuoka/73, Kanagawa/74 and GPE (–) which cross-reacted with anti-BVDV sera to some extent (19). Not only CSFV but also other pestivirus species are known to cross-react in conventional serological tests using antisera obtained from affected animals. The Japanese isolates of CSFV were further divided into six distinct (types I to VI) using 20 monoclonal antibodies prepared against CSFV strains (26). The monoclonal antibodies enabled a subdivision of the group B strains into two types, namely: V (Osaka/71, Shizuoka/73, Kanagawa/74) and VI (GPE–) and group H strains into three types: type I (Hokkaido/66), type III (Yamanashi/69, Fukuoka/72) and type IV (Ibaraki/66). Type II defined by these monoclonal antibodies contained the Nakamura/66 and Miyazaki/81 strains. Some similarities were evident with the typing based on secondary structure. Group H corresponded to subgenotype CSFV-1.1 and group B, with the exception of strains GPE (–) and Kanagawa/74, corresponded to genotype CSFV-1.2. The allocation of strain Kanagawa/74 to group B could be related to the genetic relatedness of genotype CSFV-3 with genotype CSFV-1.2. The differences in clinical and pathological aspects between CSFV subgenotypes CSFV-1.1 and CSFV-1.2 were demonstrated further in relation to strains responsible for previous and recent outbreaks in Europe (5), indicating that genetic typing of CSFV is absolutely essential. However, the molecular characterisation of CSFV isolates (e.g. by cycle sequencing of polymeraise chain reaction [PCR] products) is now favoured for classification instead of the use of monoclonal antibody panels (10, 15). Because of such reasons, PCR detection of CSFV is a well suited flanking method to supplement classical methods, such as immunofluorescence and non-cytopathogenic virus isolation as indicated by the poly(lactide) (PLA) technique.

The PNS method provides a clear picture of species and genotype boundaries, due to the exclusive consideration of strategic and highly conserved regions and consequently helps to avoid unclear classification, since palindromic nucleotide substitutions in base pairings correspond to radical evolutionary changes which can generate new species or genotypes. The results of the PNS method are essentially qualitative. However, the method applies a combination of qualitative and quantitative estimation of genomic variations providing genetic markers for the genotyping procedure and percentage values for homology among species and genotypes. The verification of relatedness between species phylogeny and segregation into genotypes supported the rationale of the PNS procedure and confirmed the reliability of the genotype identification keys and the results provided by the method. Evaluation of nucleotide base pair divergence among genotypes led to the construction of a phylogenetic tree based on secondary structure. Values of divergence among CSFV

genotypes, according to changes in nucleotide base pairs, through their mean values, provided clear indications on relatedness among genotypes and divergence in the species. At genotype level, values expressed the heterogeneity among strains in genotypes. Homology among genotypes changed with low values indicating strong correlations and higher values indicated a range of divergence among genotypes reaching marked genetic distance. The phylogenetic tree, constructed according to the mean values of base pair divergence, indicated the correlations among genotypes identifying two main branches in the species related to genotypes CSFV-1 and CSFV-2. The location of base pair changes in the three variable loci were identified for each evolutionary step generating the different genotypes. The relatedness among genotypes was characterised by shared base pairs and the occurrence of further stable mutations. Mean values of divergent base pairs indicated and quantified the genetic relatedness between genotypes in the species. These values reflected nucleotide characteristics in the specific base pairs, which were either shared by related genotypes or divergent, indicating the genetic distance between them. Stable mutation characteristics for each genotype were identified at the different variable loci levels and gave rationale to relatedness among genotypes in the species. Subgenotype CSFV-1.1 showed specific relatedness with subgenotype CSFV-1.4. Subgenotype CSFV-1.2 showed genetic links with subgenotype CSFV-1.3 and CSFV-2 and CSFV-3 genotypes, suggesting their hypothetical origin. The phylogenetic analysis based on the secondary structure was previoulsly proposed for BVDV-1 species (M. Giangaspero and R. Harasawa, unpublished findings) showing the rational approach of the PNS method providing reliable homology values and divergence determination among genotypes.

Conclusions

The application of the PNS method is an appropriate approach for differential diagnosis to solve cross-infections which may obscure the rationale for the definition of the *Pestivirus* species according to their animal host. Nomenclature for *Pestivirus* species is predominantly dependent on the animal host species from which they were isolated. There is extensive antigenic cross-reactivity among the species and they can cross the host species barrier and infect various animal species. Clinical diagnosis of CSF has sometimes been hampered because pigs affected with other pestiviruses reveal symptoms resembling CSF (27, 32, 33). It is, therefore, important to establish a reliable diagnostic procedure to distinguish CSF from other pestivirus infections in pigs. Similarly, the first report of an ovine CSFV strain (18) underlined the necessity for particular caution in the aetiological determination of clinical cases of Border disease. Previous studies have showed that BVDV-1a, BVDV-1b and BVDV-2d were related to virus circulation in sheep and were limited to Europe, and that BVDV-2b was restricted to the United States (7, 8). Strains were isolated from animals suffering from Border disease demonstrating typical symptoms caused by BDV, thus causing confusion in diagnosis.

Our results in this study suggest that the Japanese strains Kanagawa/74 along with Okinawa/86 should be classified into the third cluster, CSFV-3, which is genetically close to BDV. Thus, these strains could be a candidate for the missing-link between CSFV and BDV, since these two species are phylogenetically related (12) and the genome of BDV is more closely related to CSFV than to BVDV genotypes (1). The ovine strain 5440/99 was assigned to a separate cluster in the species, genotype CSFV-2, in respect to nomenclature reflecting the divergence within the species. The result corresponded to a primary sequence analysis, indicating that the ovine strain is a separate cluster within the species (18). These strains might be an expression of differences between geographic origins or host animal species.

Tha aim of this study was to contribute to the creation of a reliable classification of CSFV. The PNS analysis in the 5′-UTR demonstrated a rationale and simple approach for viral investigations. Secondary structures predicted

at the three variable regions in the 5′-UTR showed typical PNS which were useful for classification of the genus *Pestivirus*. Due to the global economic importance of these viruses and the difficulties encountered in the control of the diseases, it is, therefore, important to understand the genetic aspects of the viruses. The identification of viral types or subtypes based on genetic changes should improve our understanding of CSFV epizootiology and might provide markers for biological differences. Genetic variation among CSFV strains within limited geographic areas is fundamental to understand their distribution and evolutionary history and to determine control measures. Similarly, observation in wild boars might reveal interesting aspects that could useful when compared to virus molecular characteristics from pigs and other susceptible domestic animals.

References

1. Becher P., Orlich M. & Thiel H.J. 1998. Complete genomic sequence of Border disease virus a pestivirus from sheep. *J Virol*, **72**, 5165-5173.
2. De Moerlooze L., Lecomte C., Brown-Shimmer S., Schmetz, D., Guiot C., Vandenbergh D., Allaer D., Rossius M., Chappuis G., Dina D., Renard A. & Martial J.A. 1993. Nucleotide sequence of the bovine viral diarrhoea virus Osloss strain: comparison with related viruses and identification of specific DNA probes in the 5′ untranslated region. *J Gen Virol*, **74**, 1433-1438.
3. Deng R. & Brock K.V. 1993. 5′ and 3′ untranslated regions of pestivirus genome: primary and secondary structure analyses. *Nucleic Acids Res*, **21**, 1949-1957.
4. Fauquet C.M., Mayo M.A., Maniiloff J., Desselberger U. & Ball L.A. 2005. Virus taxonomy. Classification and nomenclature of viruses. Elsevier, Academic Press, San Diego, 988-992.
5. Floegel-Niesmann G., Bunzenthal C., Fischer S. & Moenning V. 2003. Virulence of recent and former classical swine fever virus isolates evaluated by their clinical and pathological signs. *J Med Vet B*, **50**, 214-220.
6. Freier S.M., Kierzek R., Jaege J.A., Sugimoto N., Caruthers M.H., Nielson T. & Turner D.H. 1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc Natl Acad Sci USA*, **83**, 9373-9377.
7. Giangaspero M. & Harasawa R. 2002. Eterogeneità dei *Pestivirus* ovini. *Vet Ital*, **38**, 59-64.
8. Giangaspero M. & Harasawa R. 2004. Genetic variety of bovine viral diarrhea virus 2 strains isolated from sheep. *J Vet Med Sci*, **66**, 323-326.
9. Grebennikova T.V., Zaberezhnyi A.D., Sergeev V.A., Biketov S.F., Aliper T.I. & Nepoklonov E.A. 1999. Genetic characteristics of the KC vaccine strain of hog cholera virus: comparative analysis of the primary sequence of surface glycoprotein E(rns), E1, and E2 genes. *Mol Gen Mikrobiol Virusol*, **2**, 34-40
10. Greiser-Wilke I., Zimmermann B., Fritzemeier J., Floegel G. & Moennig V. 2000. Structure and presentation of a world wide web database of CSF virus isolates held at the EU reference laboratory. *Vet Microbiol*, **73**, 131-136.
11. Harasawa R. 1994. Comparative analysis of the 5′ non-coding region of pestivirus RNA detected from live virus vaccines. *J Vet Med Sci*, **56**, 961-964.
12. Harasawa R. 1996. Phylogenetic analysis of *Pestivirus* based on the 5′ untranslated region. *Acta Virol* **40**, 49-54.
13. Harasawa R. & Giangaspero M. 1998. A novel method for pestivirus genotyping based on palindromic nucleotide substitutions in the 5′-untranslated region. *J Virol Methods*, **70**, 225-230.
14. Harasawa R. & Giangaspero M. 1999. Genetic variation in the 5′ end and NS5B regions of classical swine fever virus genome among Japanese isolates. *Microbiol Immunol*, **43**, 373-379.
15. Harding M.J., Prud'homme I., Gradil C.M., Heckert R.A., Riva J., McLaurin R., Dulac G.C. & Vydelingum S. 1996. Evaluation of nucleic amplification methods for the detection of hog cholera virus. *J Vet Diagn. Invest*, **8**, 414-419.
16. Higgins D.G., Bleasby A.J. & Fouchs R. 1992. Clastal V: improved software for multiple sequence alignment. *Comp Appl Biol Sci*, **8**, 189-191.
17. Hofmann M. & Bossy S. 1998. Classical swine fever in 1993 in Switzerland: molecular epidemiologic characterization of the virus isolate. *Schweiz Arch Tierheilkd*, **140**, 365-370.

18. Hurtado A., Garcia-Perez A.L., Aduriz G. & Juste R.A. 2003. Genetic diversity of ruminant pestiviruses from Spain. *Virus Res*, **92**, 67-73.
19. Kamijo Y., Ohkuma S.-I., Shimizu M. & Shimizu Y. 1977. Differences in pathogenicity and antigenicity among hog cholera virus strains. *Natl Inst Anim Health Q*, **17**, 133-140.
20. Laddomada A., Patta C., Oggiano A., Caccia A., Ruiu A., Cossu P. & Firinu A. 1994. Epidemiology of classical swine fever in Sardinia: a serological survey of wild boar and comparison with African swine fever. *Vet Rec*, **134**, 183-187.
21. Lowings P., Ibata G., Needham, J. & Paton D. 1996. Classical swine fever virus diversity and evolution. *J Gen Virol*, **77**, 1311-1321.
22. Meyers G., Rümenapf T. & Thiel H.J. 1989. Molecular cloning and nucleotide sequence of the genome of hog cholera virus. *Virology*, **171**, 555-567.
23. Meyers G., Sallmüller A. & Büttner M. 1999. Mutations abrogating the Rnase activity in glycoprotein E^{rns} of the pestivirus classical swine fever virus lead to virus attenuation. *J Virol*, **73**, 10224-10235.
24. Moormann R.M.J., Warmerdam P.A.M., van der Meer B., Schaaper W.M.M., Wensvoort G. & Hulst M.M. 1990. Molecular cloning and nucleotide sequence of hog cholera virus strain Brescia and mapping of genomic region encoding envelope protein E1. *Virology*, **177**, 184-198.
25. Moormann R.J.M., van Gennip H.G.P., Miedema G.K.W., 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.
26. Nishimori T., Yamada S. & Shimizu M. 1996. Production of monoclonal antibodies against classical swine fever virus and their use for antigenic characterization of Japanese isolates. *J Vet Med Sci*, **58**, 707-710.
27. Paton D.J., Simpson V. & Done S.H. 1992. Infection of pigs and cattle with bovine viral diarrhea virus on a farm in England. *Vet Rec*, **131**, 185-188.
28. Ruggli N., Moser C., Mitchell D., Hofmann M. & Tratschin J.D. 1995. Baculovirus expression and affinity purification of protein E2 of classical swine fever virus strain Alfort/187. *Virus Genes*, **10**, 115-126.
29. Sakoda Y., Ozawa S., Damrongwatanapokin S., Sato M., Ishikawa K. & Fukusho A. 1999. Genetic heterogeneity of porcine and ruminant pestiviruses mainly isolated in Japan. *Vet Microbiol*, **65**, 75-86.
30. Sneath P.H.A. & Sokal R.R. 1973. Numerical taxonomy: the principles and practice of numerical classification. W.H. Freeman, San Francisco, 573 pp.
31. Stadejek T., Warg J. & Ridpath J.F. 1996. Comparative sequence analysis of the 5′ non-coding region of classical swine fever virus strains from Europe, Asia, and America. *Arch Virol*, **141**, 771-777.
32. Terpstra C. 1991. Hog cholera: an update of present knowledge. *Br Vet J*, **147**, 397-406.
33. Terpstra C. & Wensvoort G. 1988. Natural infections of pigs with bovine viral diarrhea virus associated with signs resembling swine fever. *Res Vet Sci*, **45**, 137-142.
34. Uttenthal A., Le Potier M.F., Romero L., De Mia G.M. & Floegel-Niesmann G. 2001. Classical swine fever (CSF) trial I. Challenge marker vaccine studies in weaner pigs. *Vet Microbiol*, **83**, 85-106.
35. Vilcek S., Stadejek T., Ballagi-Pordany A., Lowings J.P., Paton D.J. & Belak S. 1996. Genetic variability of classical swine fever virus. *Virus Res*, **43**, 137-147.
36. Vilcek S. & Belak S. 1997. Organization and diversity of 3′-noncoding region of classical swine fever virus genome. *Virus Gene*, **15**, 181-186.
37. Widjojoatmodjo M.N., van Gennip H.G., de Smit A.J. & Moormann R.J. 1999. Comparative sequence analysis of classical swine fever virus isolates from the epizootic in The Netherlands in 1997-1998. *Vet Microbiol*, **66**, 291-299.
38. Zuker M. & Stiegler P. 1981. Optimal computer folding of large RNA sequences using thermodynamics and auxiliary. *Nucleic Acids Res*, **9**, 133-148.

