Genetic Variation in the 5’ End and NS5B Regions of Classical Swine Fever Virus Genome among Japanese Isolates

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Abstract: Sixteen clinical strains of classical swine fever virus (CSFV) isolated in Japan were subjected to analyses of nucleotide sequence variations in the 5’ end and NS5B regions of the genome. These isolates were divided into three genovars, CSFV-1, CSFV-2 and CSFV-3, based on palindromic nucleotide substitutions at the three variable loci in the 5’ untranslated region (UTR). Phylogenetic trees constructed from nucleotide sequences in the 5’-UTR and NS5B gene indicated that the CSFV strains were divided into three clusters, I, II and III. CSFV strains included in clusters I, II and III were identical to those in the CSFV-1, CSFV-2 and CSFV-3 genovars, respectively.

Key words: Classical swine fever virus, Hog cholera, Pestivirus

Classical swine fever virus (CSFV; synonym of hog cholera virus or pestivirus), a causative agent of swine fever or hog cholera, is a species, along with border disease virus (BDV) and bovine [viral] diarrhea virus (BVDV), of the genus Pestivirus within the family Flaviviridae. The CSFV genome is a single-stranded positive-sense RNA which is bracketed by untranslated regions (UTR) at the 5’ and 3’ ends (3).

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 (12, 24, 25). Group I comprises the reference strain Brescia together with old American, European and Japanese isolates, and group II includes the reference strain Alfort and recent European isolates. The Japanese isolates examined so far have been classified into group I only (12, 18, 25) except for the Osaka/71 strain, which was allegedly isolated from a pig during quarantine shortly after importation from the UK in 1971 (9). The Japanese strain Kanagawa/74, isolated in 1974, has been considered as a disparate strain among CSFV strains according to phylogenetic analyses (12, 25). Therefore, it is of interest to examine genetic variations among the CSFV strains isolated in Japan so as to know whether or not there exist group II strains among the Japanese isolates. In the present study, we sequenced the 5’-UTR and the 3’ end of the viral genome, including a partial region of the NS5B gene and the 3’-UTR, and compared the primary and secondary structures of these regions with other pestiviruses.

Materials and Methods

Virus strains. Sixteen strains of CSFV isolated between 1951 and 1986 in Japan were obtained from the National Institute of Animal Health, Tokyo. They are Hokkaido/66, Nakamura/66, Miyazaki/81, Yamanashi/69, Ibaraki/66, Osaka/51, Shizuoka/73, Kanagawa/74, Saitama/81, Okinawa/86, Fukuoka/72, Osaka/71, Ibaraki/81-
115, Ibaraki/81-20, Ibaraki/81-38, and Ibaraki/81-40. Numbers after the slash indicate the year of isolation. The Osaka/71 and Fukuoka/72 strains, out of these 16 strains, were allegedly isolated from pigs revealing typical signs of hog cholera at the animal quarantine station at the time of importation from England and Korea, respectively (9). The 14 other strains of CSFV were clinical isolates from outbreaks of hog cholera in Japan. A modified-live vaccine strain, GPE-, was recovered from a commercial CSFV vaccine lot (Matsukens Pharmaceutical Co., Ltd., Tokyo).

**Extraction of pestivirus RNA from virus cultures.** Ribonucleic acids were isolated from each virus culture by the single-step guanidinium isothiocyanate-phenol-chloroform method (1) using a RNAzo-1 extraction kit (Biotecx Laboratories, Inc., Houston, Tex., U.S.A.). Briefly, 200 µl of each virus culture fluid was mixed vigorously with 800 µl of a solution containing guanidium isothiocyanate-phenol in a 1.5-ml Eppendorf tube. One-hundred microliters of chloroform was added and the tube was gently vortexed for 30 sec by hand. The tube was chilled on ice for 5 min and then spun in an Eppendorf centrifuge for 10 min. The aqueous phase (ca. 600 µl) was transferred into a fresh Eppendorf tube. One microliter of 20 mg/ml mussel glycogen (Boehringer Mannheim GmbH, Germany) and 600 µl of isopropanol were added and the tube was chilled on ice for 30 min. The RNA precipitate was collected by centrifugation for 10 min. The pellet was washed three times with 600 µl of 75% ethanol. The pellet was air-dried, dissolved in 16 µl of sterile distilled water treated with 0.1% diethyl pyrocarbonate (DEPC), and then heated at 60 C for 10 min. Eight-microliter aliquots of RNA solution were subjected to reverse transcription reaction.

**Oligonucleotides.** Oligonucleotide primer sequences of the 5'UTR were based on genomic sequences of high homology among CSFV strains reported previously. The first strand of the 5'-UTR of CSFV cDNA was synthesized using an oligonucleotide primer R1 (5'-ACTCCATGTGCCATGTACAG-3') and R1 are described elsewhere (6). Their equivalent locations in the Alfort strain of CSFV are FI = 90-109 and R1 = 351-370. Oligonucleotide primers 3E1 (located in the genome of strain Alfort: 11906-11929) and 3E2 (located in the genome of strain Alfort: 12245-12264), for amplification of the 3' terminal region including the NS5B region, were the same as described by Vilcek et al (24). All oligonucleotides were custom-made by Takara Shuzo Co., Ltd. (Kyoto, Japan).

**Reverse transcription (RT) reaction.** The cDNA synthesis and polymerase chain reaction (PCR) were performed as described previously (6). To 8 µl of each RNA solution were added 8 µl of 5X buffer and 4 µl of 0.1 M dithiothreitol (both supplied with the enzyme), 0.25 µl (25 units) of reverse transcriptase M-MLV (Gibco-BRL, Gaithersburg, Md., U.S.A.), 8 µl of deoxyribonucleotide triphosphates (dNTPs) to a final concentration of 0.2 mM each, 0.2 µl (110 U/µl) of ribonuclease inhibitor (Takara Shuzo Co., Ltd.), 0.25 µl of 3' downstream primer (40 pmol/µl), and DEPC-treated water to a final volume of 40 µl. The first strand synthesis of cDNA was carried out at 37 C for 90 min. A 5-µl portion of this reaction mixture was used directly for the PCR assay.

**PCR.** The PCR amplification was carried out with 50-µl reaction mixtures containing 5 µl of cDNA solution, 5 µl of 10X buffer, 1.25 U of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn., U.S.A.), dNTPs to a final concentration of 0.2 mM each, 0.25 µl of each pair of 5' upstream and 3' downstream primers (40 pmol/µl each), and water to a final volume of 50 µl. After the mixture was overlaid with mineral oil, the reaction cycle was carried out 30 times with denaturation at 94 C for 30 sec, annealing at 56 C for 120 sec, and extension at 72 C for 120 sec.

**Agarose gel electrophoresis.** The PCR products were fractionated using agarose gel electrophoresis and visualized by staining with ethidium bromide. Ten-microliter aliquots of the PCR products were mixed with 2 µl of 6X dye solution consisting of 0.25% xylene cyanol, 0.25% bromophenol blue and 40% sucrose in water, run on horizontal, submerged 2.0% NuSieve 3:1 agarose gels (FMC Bioproducts, Rockland, Me., U.S.A.) in TAE (40 mm Tris, pH 8.0, 5 mm sodium acetate, 1 mm disodium EDTA) buffer at 50 volts for 50 min, and stained with ethidium bromide (0.4 µg/µl) for 15 min.

**Analysis of the nucleotide sequences.** Clearly visible bands of correct size for the primer pairs in the PCR were extracted from the gels, and were subjected to direct sequencing three times on each strand in an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Cetus Corp.). Nucleotide sequences in the 5' end and NS5B regions of the CSFV genome were aligned by the method of Higgins et al (7) using the DNASIS program package (Hitachi Software Engineering Co., Yokohama, Japan). Secondary structures were predicted according to the algorithm of Zuker and Stiegler (27), and compared with those of BVDV and BDV. The folding energies were calculated by the method of Freier et al (4). The variable regions in the 5'-UTR were subjected to palindromic nucleotide substitutions (PNS) analysis (5). The phylogenetic tree-based nucleotide sequences in the 5'-UTR and NS5B region were constructed by the unweighted pair-group method using arithmetic averages (UPGMA) of Sneath and Sokal (19). The nucleotide sequences of relevant regions were obtained from the published data for reference.
pestivirus strains, Alfort (14), Brescia (15) and Osloss (2). Nucleotide sequences of the other pestivirus strains, obtained from the DNA databases, are as follows (accession number is given in parentheses): 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 EBr (D50817), 890 (L32886) and CD87 (L32887) for BVDV-2; and strains BD31 (U70263), Moreduin (U65023) and Ch1Es (D50816) for BDV.

Nucleotide sequence accession numbers. The nucleotide sequences of the CSFV cDNA first presented in this paper have been deposited in the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under the following accession numbers: AB019149 to AB019182.

Results

The 5' and 3' terminal regions of the 17 CSFV strains were PCR-amplified and sequenced by using the primers described in "Materials and Methods." The PCR products of each region were approximately 285 and 350 base-pairs for the 5' and 3' terminal regions, respectively.

A small number of nucleotide substitutions were apparent at three variable regions, V1, V2 and V3, in the 5'-UTR of the CSFV strains (Fig. 1). Each variable region contains a palindromic nucleotide sequence which allows the formation of a stem-loop structure. A stem-loop structure predicted at the V1 region consisted of 26 nucleotides including a bulge loop of C and C pairing at position five from the bottom. A loop structure at the V2 region was characterized by a quintuplet nucleotide, 5'-GGGGU-3'. According to the PNS which was responsible for alteration of a secondary structure at the three variable regions, the CSFV strains were divided into three genovars, CSFV-1, CSFV-2 and CSFV-3 (Fig. 2). CSFV-1 was composed of strains Brescia, Hokkaido/66, Nakamura/66, Yamanashi/69, Fukuoka/72 and Miyazaki/81. CSFV-2 was composed of strains Alfort, Osaka/51, Osaka/71, Ibaraki/66, Ibaraki/81-20, Ibaraki/81-38, Ibara-
ki/81-40, Ibaraki/81-115, Shizuoka/73 and Saitama/81. CSFV-3 was composed of strains Kanagawa/74 and Okinawa/86. A mis-matched base-pairing by A to C due to non-complementary transition from U, which was distinct from two other genovars, was evident at the bottom of a stem-loop structure for the V2 region of CSFV-1. CSFV-3 strains showed PNS in the V1 and V3 regions. A Watson-Crick base-pairing of A-U at position nine from the bottom of a stem-loop structure for the V1 region of CSFV-3 was distinct from the other genovars of CSFV in terms of transitional substitution from C to U. Additionally, a mis-matched pairing by A and G at the bottom of a stem-loop structure, which was based on 

Fig. 2. Secondary structures predicted at the three variable regions, V1, V2 and V3, in the 5'-UTR of CSFV strains. Watson-Crick base pairing is shown by a dash (—), and G to U pairing tolerated in secondary structures is shown by an asterisk (*). CSFV strains were divided into three genovars, CSFV-1, CSFV-2 and CSFV-3, according to the PNS. Differential base-pairings for genovars are enclosed in squares.

Fig. 3. Phylogenetic tree constructed from the 5'-UTR of CSFV strains, together with BDV and BVDV strains, according to the UPGMA. Strain name is given in parentheses. The numbers at the nodes of branches are bootstrap values obtained from 1,000 replications. The numbers at the branches indicate Euclidean distance.
transversal nucleotide substitution from U to G, was characteristic to the V3 region of CSFV-3.

A phylogenetic tree constructed from the 5'-UTR of the CSFV strains along with other pestiviruses using the UPGMA indicated that the CSFV strains were divided into three groups, I, II and III (Fig. 3). Strains included in groups I, II and III were identical to those in the CSFV-1, CSFV-2 and CSFV-3 genovars defined by the PNS at the three variable regions, respectively. Group II included not only the Osaka/71 strain, which has been considered as an alien strain from England, but also the Osaka/51, Shizuoka/73, Saitama/81, Ibaraki/81-115, Ibaraki/81-20, Ibaraki/81-38, and Ibaraki/81-40 strains isolated from outbreaks of hog cholera in Japan.

A phylogenetic tree, constructed from the nucleotide sequences at the NS5B gene of CSFV according to the UPGMA. The numbers at the nodes of branches are bootstrap values obtained from 1,000 replications. The numbers at the branches indicate Euclidean distance.

**Discussion**

Classical swine fever (CSF) is of world-wide economic importance, and is generally subject to statutory control involving slaughter of affected pigs and restrictions on the movement of pigs from an affected area. Despite intensive eradication efforts, CSF has recurred in many parts of the world. Wild boar has been suspected as an important reservoir of CSFV on the European Continent (11). Clinical diagnosis of CSF has sometimes been hampered, because pigs affected with other pestiviruses reveal symptoms resembling CSF (17, 22, 23). It is, therefore, important to establish a reliable diagnostic procedure to discriminate CSF from other pestivirus infections in pigs.

Clinical isolates of CSFV from outbreaks of hog cholera in Japan have been divided into two groups, H and B, on the basis of differences in pathogenicity revealed by experimental infections as well as differences in reactivity with polyclonal anti-BVDV immune sera prepared in pigs. Group H (CSFV which causes a typical form of hog cholera) comprised CSFV strains Hokkaido/66, Ibaraki/66, Yamanashi/69 and Fukuoka/72, which did not react with anti-BVDV sera; and group B (BVDV-related CSFV which causes a chronic type of illness) has comprised strains Osaka/71, Shizuoka/73, Kanagawa/74 and GPE- which cross-reacted with anti-BVDV sera to some extent (9). 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 have been further divided into six distinct types, I to VI, using 20 monoclonal antibodies prepared against CSFV strains (16). The monoclonal antibodies have allowed the subdivision of group B strains into two types, V (Osaka/71, Shizuoka/73, Kanagawa/74) and VI (GPE-) and of group H strains into three types, I (Hokkaido/66), III (Yamanashi/69, Fukuoka/72) and IV (Ibaraki/66). Type II defined by these monoclonal antibodies has contained the Nakamura/66 and Miyazaki/81 strains.

Recently, the application of RT-PCR-based methods became available for the diagnosis of pestivirus infections (8, 10, 13, 26). These molecular methods allow division of the CSFV strains into at least two genetic groups, I and II (12, 20, 21). The CSFV strains isolated from hog
References


