Molecular Evolutionary Analysis of Classical Swine Fever Virus

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ABSTRACT

The E2 glycoprotein gene of cases of 15 classical swine fever virus (CSFV) were isolated from HeiLongJiang province of China from 2011 to 2012 were amplified by RT-PCR. A 190 nt amplicon of E2 gene was sequenced and compared with 7 CSFV reference strains. Phylogenetic analysis of 190 nucleotides of E2 gene indicated that 15 HeiLongJiang isolates was classified into subgroup 1.1, which indicated that the CSFV Group 1 were still contributing to the epidemic of classical swine fever (CSF) in HeiLongJiang province of China. We synchronously performed Bayesian Markov chain Monte Carlo (MCMC) analysis of the E2 gene region to investigate the diversity of evolutionary rates between Group 1 and Group 2 of CSFV. The results revealed that the mean evolutional rate of Group 1 and 2 were 5.88×10^{-4} and 3.04×10^{-3} substitutions per site per year, respectively, which demonstrated that Group 2 evolved much faster than Group 1. The striking differences in evolutionary rates of two genotypes likely implied that both of them had their different evolutionary strategies.

Key Words: Classical swine fever virus, Envelope glycoprotein E2, Molecular evolutionary.

INTRODUCTION

Classical swine fever (CSF) caused by classical swine fever virus (CSFV) is a highly contagious disease of pigs and wild boars. CSFV belong to the *Pestivirus* genus within the *Flaviviridae* family (1). CSFV is an enveloped virus with a single-stranded RNA genome of about 12.3kb in full length, which consists of 5´- and 3´- untranslated regions (UTR) flanking a single large open reading frame (ORF) coding for a polyprotein of about 3898 amino acids (2). The polyprotein is processed to four structural proteins (C, E^{rns}, E1, E2) and eight non-structural (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (3). As one of the major structural glycoproteins, E2 protein, molecular weight of 51-55kD and 373 amino acid residues (4), always exists on the surface of viral particles and is the most immunogenic among the CSFV proteins. E2 can not only induce neutralizing antibodies with high titer, but also plays a decisive role in cell tropism and virulence of CSFV's (5). In the variable N-terminal of E2 protein, the 190 nt located in 2518-2707 is extensively used for the genotyping and genetic comparison of CSFV's (6). Previous studies on the phylogenetic relationship of CSFV's have divided the viruses into three main groups, and indicated that all the isolates in mainland China belonged to Group 1 and 2.

It is well known that the Group 1 viruses consist of highly virulent strains which could cause acute CSF, while the Group 2 viruses are characterized by moderately virulent strains which caused the subacute and chronic CSF (7, 8). However, the genetic diversity of different genotypes of CSFV remains elusive, and the studies on their evolutionary rates are few.

In the present study, based on the analysis of the 190 nt E2 sequence, newly sequenced E2 genes of 15 CSFVs isolated from HeiLongJiang province were investigated for phylogenetic relationship and their substitution rates of the CSFV's within Group 1 and Group 2.

MATERIALS AND METHODS

Virus isolates

The 15 new CSFVs were obtained from tissue samples of sick pigs (n=100) in different herds in HeiLongJiang province of China from 2011 to 2012. Seven reference virus strains were retrieved form GeneBank website (http://www.ncbi.nlm.nih.gov/) and EU reference laboratory for CSFV database in Hannover (http://viro08.tiho-hannover.de/eg/csf/startCSF.cgi). Beside the 15 sequenced isolates in this study, total of 34 isolates (Group 1) and 58 isolates (Group 2) from mainland China were collected from 1982 to 2009 and geographically characterized from all around of China for evolutionary rate analysis.

RNA isolation, RT-PCR and sequencing

Total RNA was extracted from homogenates of CSFV infected lymph nodes and spleen tissues using Trizol reagent (Invitrogen, USA) according to the manufacture's recommendations (9). The sense primer 5′-TCRWCAACCAAYGAGATAG-3′ and the antisense primer 5′-CACAGYCCRAAYCCTAAGTCA-3′ was chemically synthesized based on a previously published study (10). After reverse transcription reaction was carried out, the PCR reaction was performed as follows according to the product's protocol. Cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 30 cycles with 94 °C for 50 sec, 50 °C for 50 sec and 72 °C for 1 min. The amplified product of 258 bp was purified and cloned into pMD18-T vector (Takara, Dalian, China) and sequenced by Shanghai Sanggo Co. Ltd, China.

Phylogenetic analyses

Bioedit software (version 7.0.1) was performed for sequence comparison of 190 nt sequence of 15 CSFV isolates with reference Shimen (U72047) and HCLV strains (U72048). Meanwhile, all isolates retrieved from GenBank database and EU reference laboratory for CSFV database were multiply aligned using Clustal X (version 1.83) (11). The DNAstar computer software package (MegAlign, version, 5.01) was used for analyzing divergence and similarity of the 15 isolate sequences (12). The software package Tree-Puzzle (version 4.0.2) was employed to estimate the value of transition/transversion ratio (13). The phylogenetic tree was constructed by the neighbor-joining (NJ) method with Phylip software package (version 3.68) (14).

Estimation of substitution rates

The E2 gene sequences of CSFV isolates were compiled and aligned. Each group of the viruses was analyzed independently. We firstly aligned sequences using software Clustal X (version 1.83) (11), and then tested the best-fitting model of nucleotide substitution for each dataset using Modeltest (version 3.7) (15). For Group 1 and Group 2 sequence datasets, the best-fitting models were determined by the Hasegawa-Kishino-Yano (HKY) model and the Hasegawa-Kishino-Yano model with Gamma (HKY+G), respectively. Bayesian Markov chain Monte Carlo approach (MCMC, the BEAST package, version 1.5.1) was used to precisely estimate the viral substitution rates (16). The common logarithm of Bayes Factors ($\log_{10}$BF) on tree likelihoods were used to choose the best-fitting models (in Tracer 1.4, http://beast.bio.ed.ac.uk/Tracer), and assumed a threshold of logBF > 2 for significance (http://beast.bio.ed.ac.uk/Model_comparison).

RESULTS AND DISCUSSION

RT-PCR and phylogenetic analysis

Envelope glycoprotein E2 gene was amplified by RT-PCR from CSFV virus. A fragment of 268 bp was obtained from the genomic DNA (Figure 1). This fragment was directly cloned in T Vector using T-A cloning method, and its nucleotide sequence was determined. The results of sequence comparison among 15 CSFV isolates are represented in Figure 2. Homogeneity assay using DNAstar software package indicated the 95.3-100% similarity among 15 CSFV isolates,

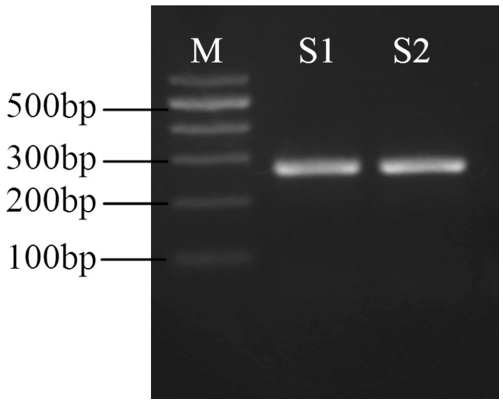

Figure 1. RT-PCR detected CSFV isolates. Lane M: DNA Marker DL2K plus; Lane S1: virus sample 1; Lane S1: virus sample 2.

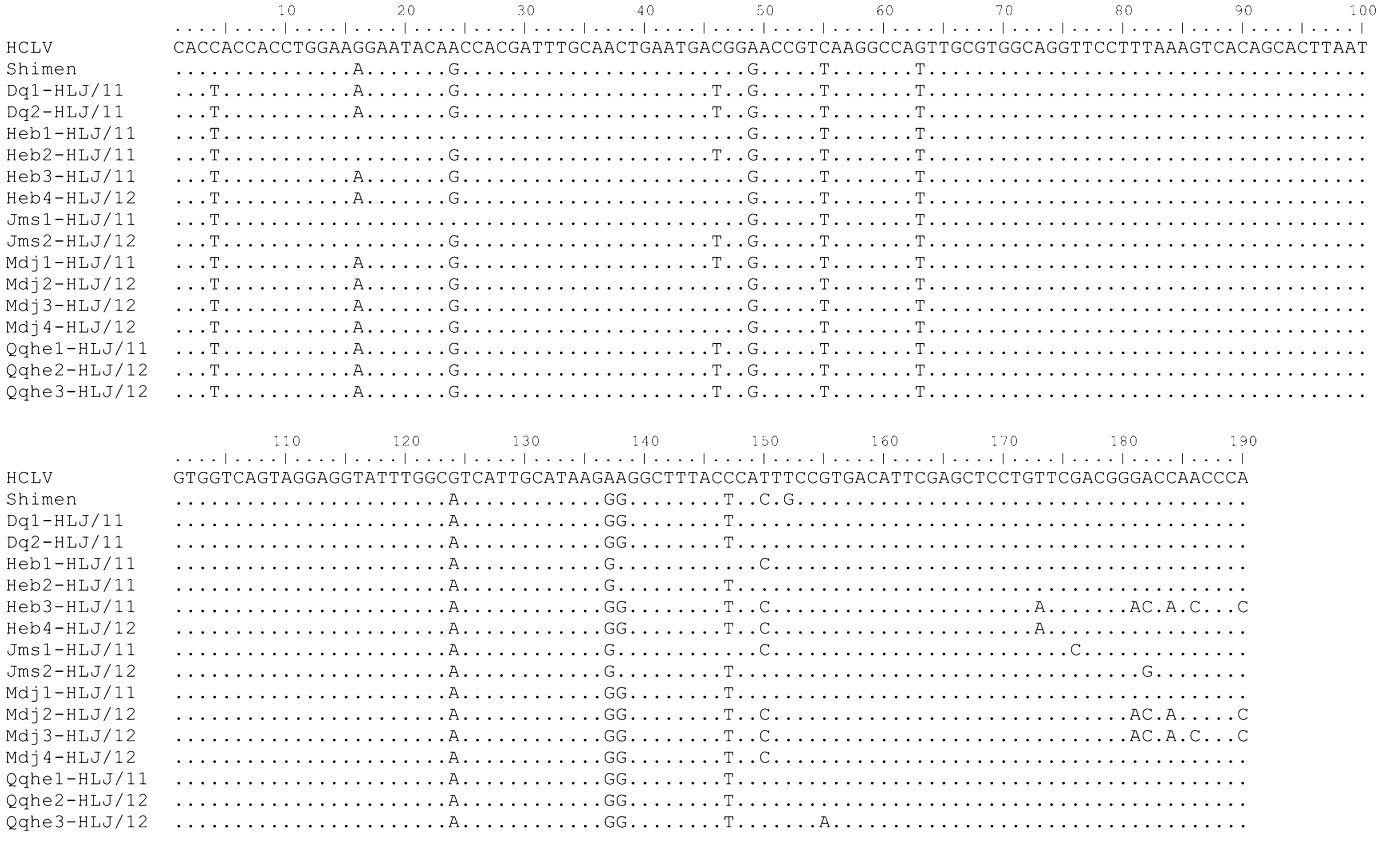

Figure 2. Alignment of 190 nt sequence of 15 CSFV isolates in this work using Bioedit software (version 7.0.1). Nucleotide Sequences shown correspond to position 2518 to 2707 within the CSFV complete genome used to derive the phylogenetic tree depicted in Figure 3. The same nucleic acid and nucleic acid deviation were indicated by dots and letters (A, T, C, G) respectively.

92.6-96.3% homology compared to the HCLV strain in the 190 nt of E2 fragment, and 97.4-98.8% homology with Shimen strain. All the 15 isolates were segregated into Group 1 only thus it became evident that Group 1 predominated in the more recent epizootics in HeiLongJiang province of China. To define the evolutionary relationships among CSFV's and genotypes of epidemic CSFV's, we referred to the phylogenetic tree of E2 sequences. All 15 CSFV isolates sequenced in this study were classified into subgroup 1.1 (Figure 3). Previous studies revealed that Group 2, especially subgroup 2.1 was the main genotype that contributed to the epidemic of CSF virus during the last decades in China (6, 17). However, our data showed that all 15 isolates sequenced in this study were classified into subgroup 1.1, which demonstrated that the total number of isolates for subgroup 1.1 was equal to subgroup 2.1. Our results suggested that CSFV subgroup 1.1 was still playing a vital role in the epidemic of CSF in some areas in China.

Rates of nucleotide substitution

The variable-rate relaxed molecular clock was determined to fit better to the data than the strict (constant) molecular clock both in Group 1 and Group 2. It is striking that Group 2 CSFV, with a mean nucleotide substitution rate of 3.04×10^{-3} substitutions per site per year (subs/site/year), was approximately five times that of Chinese Group 1, with a mean substitution rate of 5.88×10^{-4} subs/site/year (Figure 4). Small increases in the RNA virus mutation rate caused the virus serious fitness effects (18), and higher substitution rates from Group 2 CSFVs suggesting the trend to switch from Group 1 to 2, which could further explain why these viruses are prevailing in many countries of the world (17). As substitution rates are determined by a combination of forces, including the viral generation time, transmission manner, intrinsic frequency of mutation per round of replication and the extent of natural selection (19), it will ultimately be necessary to document the contribution

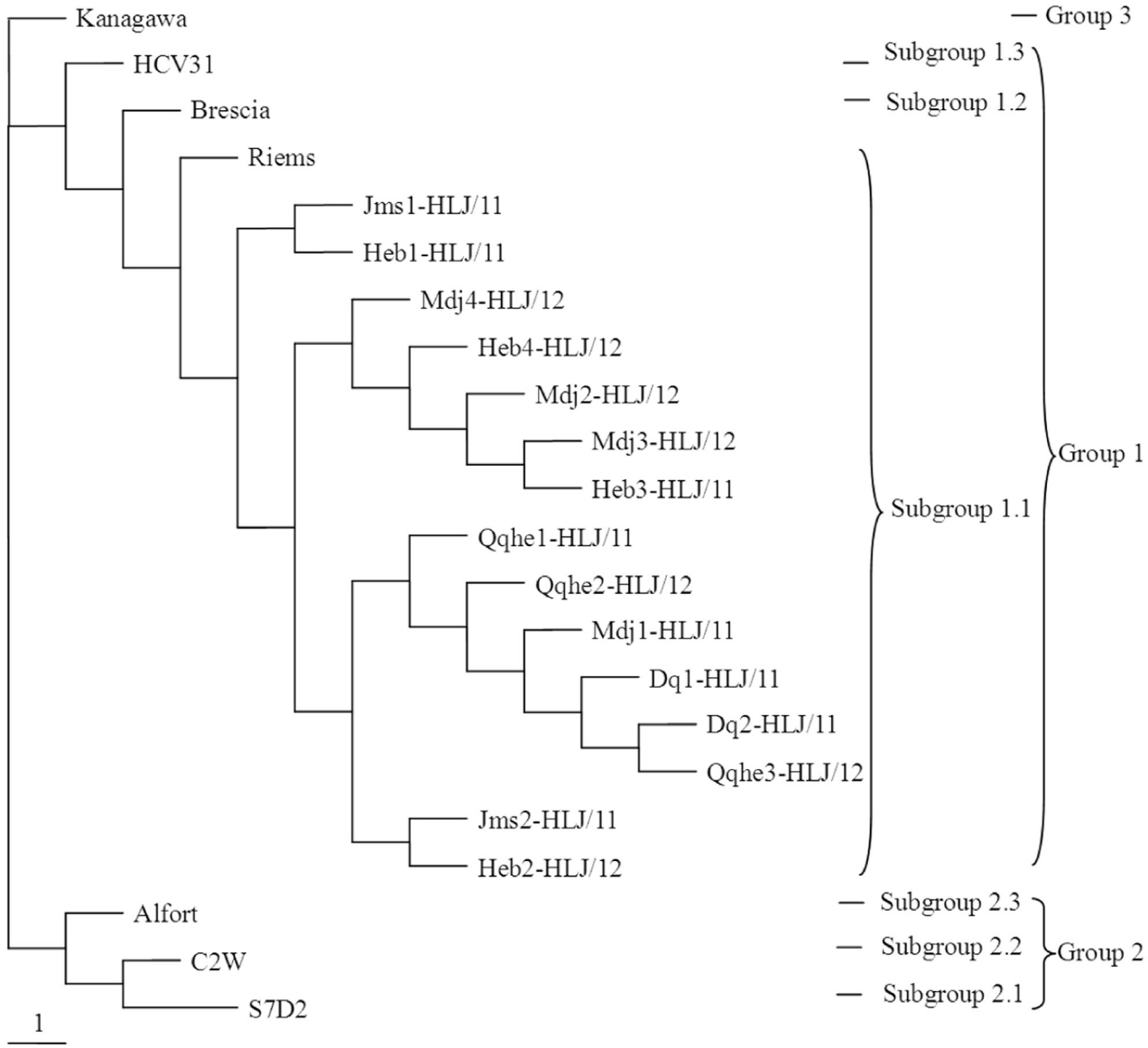

Figure 3. Neighbor-joining method of phylip was for construction of phylogenetic tree on the basis of E2 gene variable region (190 nt). Seven reference strains (Riems/Germany, U45277, subgroup 1.1; Brescia/Italy, M31768, subgroup 1.2; HCV31/Honduras, AJ781098, subgroup 1.3; S7D2/Italy, L36171, subgroup 2.1; C2W/Italy, M36165, subgroup 2.2; Alfort/Germany, J04358, subgroup 2.3; Kanagawa/Japan, subgroup 3.4) and 15 new CSFV isolates in current study were analyzed. Transition/transversion was estimated 5.52 with Tree-puzzle software. Bootstrap values were estimated for 100 replicates, and Kanagawa was treated as outgroup. Group and subgroup are shown in the right part of the corresponding grouping.

of each to the high rates of evolutionary changes seen in CSFV Group 2. It has been reported that Group 2 CSFVs were responsible for the rising incidence of subacute and chronic CSF outbreaks (7, 20), which could suggest that Group 2 viruses have to optimize the evolutionary rate under the constant selection pressure to escape the host immune response.

In brief, we report that the genotype 1.1 of CSFVs is still prevailing in HeiLongJiang province in China. Evolutionary analysis demonstrated that the Group 2 CSFVs evolved faster than the Group 1 viruses, which suggested that the immune selection pressure may be responsible for difference of CSFV evolutionary rates.

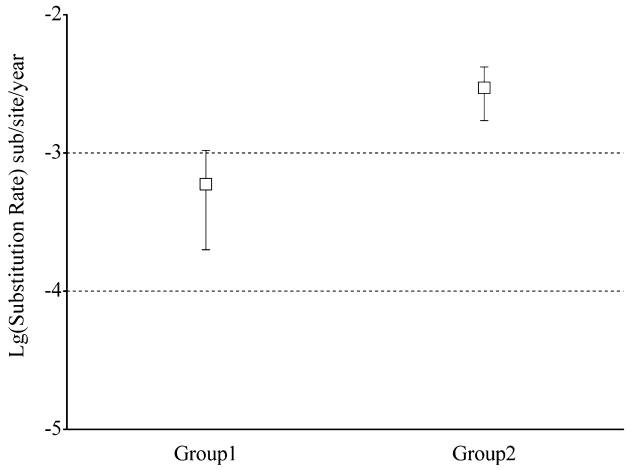

Figure 4. Average rates of nucleotide substitution of E2 genes of CSFV's estimated from the datasets collected from different regions. The hollow rectangles and straight lines represented the mean rates with the 95% highest probability densities (HPD). The data sets of Group1 and Group2 have dates of isolation ranging from 1982-2009, 1984-2008.

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