Characterization and evolutionary analyses of Pestivirus A isolated from cattle in China

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Abstract

Background Bovine viral diarrhea virus (BVDV) is a single stranded RNA virus with worldwide distribution that belongs to the genus *Pestivirus*, family *Flaviviridae*. Infection of cattle with BVDV causes a range of clinical syndromes and can result in major economic losses. Here, we reported two BVDV isolates obtained from serum collected from cattle in Shandong province, China and commercial fetal bovine serum. **Results** The two BVDV isolates, named BVDV BJ-2013 and BVDV BJ-2016, were both the members of *Pestivirus A*, belonged to genotype 1a and 1b, respectively. BVDV BJ-2013 was an cytopathogenic biotype virus, meanwhile BVDV BJ-2016 was non-cytopathogenic biotype virus. The two isolates, both with 45.3% GC content, were 12,305 nt (BVDV BJ-2013) and 12,273 nt (BVDV BJ-2016) in length. Phylogenetic analyses showed that BVDV BJ-2016 was genotype 1B of *Pestivirus A* and closely related to isolates from South Korea (JQ418634) and the US (JN380088). Simultaneously, BVDV BJ-2013 was genotype 1A of *Pestivirus A* and related to isolates from the USA (AF091605) and Germany (AF041040). Recombination analysis showed that BVDV isolate (KJ541471) was a mosaic of sequences derived from two isolates of the genotype 1A. Evolutionary analysis suggested that genotype 1A and 1B had similar relative rates and the dN/dS ratios. Comparison analysis of amino acid sequence indicated that there were 12 amino acids (EGNWLVNADRLM) insertion in NS2 of BVDV BJ-2013. **Conclusion:** In present study, two BVDV isolates were isolated and identified. Phylogenetic analyses and evolutionary analysis suggested that BVDV strains isolated from different regions showed close evolutionary relationships. It also reminded us again that the risk of trans-regional transmission of BVDV and BVDV contamination in commercial fetal bovine serum were what we must not neglect.

Background

Bovine viral diarrhea virus (BVDV) is a globally-distributed virus that is responsible for numerous clinical disease syndromes in cattle that can lead to major economic losses [1]. The virus is a member of the genus *Pestivirus*, family *Flaviviridae*. In 2017, revision to the taxonomy of the genus *Pestivirus* was performed in the ICTV tenth report [2–3]. Based on the phylogenetic analysis of amino acid sequences of the conserved regions, the genus *Pestivirus* was divided into 11 groups (species) and a new uniform naming system for species with the format *Pestivirus X* was proposed, which was a host-independent manner[3]. BVDV was divided into two species, including *Pestivirus A* and *Pestivirus B*, formerly known as BVDV 1 and BVDV 2[3]. Due to the genetically heterogeneous of BVDV, both two species are divided into multiple genotypes[4]. *Pestivirus A* is divided at least seventeen genotypes, meanwhile *Pestivirus B* is divided into four genotypes. Both two BVDV species have two biotypes, including non-cytopathogenic biotype (NCP) and cytopathogenic biotype (CP).

In many parts of the world, BVDV causes high production losses in cattle due to its adverse effects on the reproductive, enteric and respiratory systems of infected animals[5]. Furthermore, fetal infection results in the production of persistently infected (PI) calves. These PI calves are considered to be an important source of ongoing, endemic, infection due to their shedding of large amounts of BVDV throughout their lives[6–8].

In China, BVDV was first isolated in 1983[9]. Since then, the virus has spread rapidly across the country. To date, bovine viral diarrhea virus or antibody has been detected in a variety of animals, such as Chinese yellow cattle, buffalo, yaks, pigs, deer, sheep and camels[10–14]. This suggests that BVDV infection is common in China and the large number of reservoir hosts poses serious challenges to measures aimed at the eradication, prevention and control of BVDV infection.

In the current study, we sought to determine the full-length genome sequences of two BVDV isolates and to analyze their evolutionary phylogenetic relationships for understanding the genetic evolution of BVDV.

Results

Virus isolation and identification

Following inoculation of MDBK cells with three blind serial passages of the virus, two of the samples were detected as BVDV positive by RT-PCR. The IF assay revealed that MDBK cells inoculated with the third passaged cell culture were BVDV positive (Fig. 1). Virus isolated from serum sample collected in 2013 was named as BVDV BJ–2013 and virus isolated from commercial fetal bovine serum was named as BVDV BJ–2016. The significant cytopathic effects (CPE) were observed in MDBK cells infected with isolate BVDV BJ–2013 (Fig. S1). There were no CPEs visible in MDBK cells infected with isolate BVDV BJ–2016. The negatively stained virus particles collected from the third passaged cell cultures were approximately 40 nm–60 nm in length, and displayed a typical BVDV morphology (Fig. S2).

Genome analysis

The two complete genomes of BVDV isolates were sequenced. The two sequences, herein as BVDV BJ–2013 (MH490942) and BVDV BJ–2016 (MH490943), were identified as having 99% homology with the nucleotide identity of various Pestivirus A strains in GenBank. The BVDV BJ–2013 isolate had a genome of 12,305 nt in length and contained a single open reading frame (ORF) that encoded a polyprotein of 3,910 amino acids starting at position 385 and ending at position 12,078. The genome was flanked by 5' and 3' UTRs located at positions 1–384 and 12,079–12,305 nt, respectively. Isolate BVDV BJ–2016 was a full length genome of 12,273 nt in length that encoded a polyprotein of 3898 amino acids starting at position 384 and finishing at position 12077. The genome was flanked by 5' and 3' UTRs, located at positions 1–383 and 11972–12,273, respectively.

Phylogenetic analysis

The maximum likelihood Phylogenetic tree inferred from the complete genomes showed that isolate BVDV BJ–2016 (identified by a white arrow in Fig. 2) was located within the clade composed of Pestivirus A strains of genotype 1B (identified by a grey rectangle in Fig. 2). This clade was the largest in the genome phylogenetic tree, which was consistent with existing data showing that, in many countries,

BVDV of genotype 1B was the most prevalent subtype[4]. Further, isolate BVDV BJ–2016 was closely related to isolates from South Korea (JQ418634) and the US (JN380088), thus corroborating predictions about the ability of subtype 1B to spread worldwide. Isolate BVDV BJ–2013 (identified by a black arrow in Fig. 2) was clustered within a group composed of Pestivirus A strains of genotype 1A. Isolate BVDV BJ–2013 was related to isolates from the USA (AF091605) and Germany (AF041040). This subtype was reportedly the second most prevalent in many countries, including in Asian countries [4]. So, the two BVDV isolates in this study, BVDV BJ–2013 and BVDV BJ–2016, were the members of Pestivirus A.

Recombination analysis of the polyprotein product of Pestivirus A

Initially, we used default parameters to screen for a recombination signal in the alignment of the 48 Pestivirus A strains. This screening process correctly identified strain KJ541471 (isolated in China in 2013) that had previously been detected by the phylogenetic analysis (indicated by a black triangle in Fig. 2). During screening, certain parameters were modified, such as the probability threshold (*P*-value cut-offs), windows and step sizes, in order to avoid false positive signals of recombination. A strong (based on *P*-values) signal of recombination was only detected when strain KJ541471 was included in the alignment. On the contrary, alignments containing all strains or exclusively genotype 1A or genotype 1B sequences did not indicate recombination, even using the upper threshold of $P < 0.05$. By doing this we were able to determine that KJ541471 was a mosaic of sequences derived from two isolates of the genotype 1A. To determine the breakpoints of the recombinant strain, an alignment containing only genotype 1A strains was used. For this step we selected the method that provided the highest probability of recombination (the maxchi method) to locate the breakpoints (Fig. 3b). The results indicated that strain KJ541471 had two breakpoints (6980/6981 and 9565/9566) in the polyprotein region. Subsequently, the polyprotein alignment was partitioned into two fragments: the first was a concatenated alignment comprising nucleotides 1 to 6980 plus nucleotides 9567 to 11682. The second fragment corresponded to nucleotides 6981 to 9566 (Fig. 3b). Maximum likelihood trees were constructed for each fragment and the results were in agreement with the predicted locations of the breakpoints because they showed that the strain KJ541471 was located in conflicting positions on the trees. In particular, the tree constructed from the concatenated fragment (Fig. 3a) indicated that strain KJ541471 was related to strain KP941584 that was isolated in 2014 from the US. On the other hand, the tree constructed with fragment 6981 to 9566 (Fig. 3c) indicated that strain KJ541471 and isolate BJ–2013 are closely related. Apart from strain KJ541471, mosaic genomes were also detected in other strains (indicated by black rectangles in Fig. 2), but these were not regarded as recombinants due to their weak signals and unclear locations of the breakpoints. These problematic strains were excluded from the analyses of evolutionary rates and the coalescent analysis.

Evolutionary rates of Pestivirus A genotype 1A and 1B

In order to explore the differences between *genotype* 1A and 1B we estimated their genetic distances based on pairwise distances. In particular, isolate BVDV BJ–2016 was compared to South Korean isolate JQ418634 (indicated by a white dot in Fig. 2). The nucleotide distance between these two isolates was, on average, 0.052 ± 0.003 . The nucleotide distances among the isolates of genotype 1B were, on average, 0.048 ± 0.001 . The same measurements were performed with strains of clade 1A. The nucleotide distance between isolate BVDV BJ–2013 and isolate AF091605 from Oregon in the US (indicated by a black circle in Fig. 2) was, on average 0.072 ± 0.002 while the overall distance among all 1A strains was 0.10 ± 0.021 . The higher mean distances observed in genotype 1A might be related to the reduced sample size. For this reason, additional analyses were performed. Two maximum likelihood methods were used to estimate the evolutionary rates in each codon of the polyprotein region of BVDV genomes. The results indicated that the relative rates (Fig. 4a) and the dN/dS ratios (Fig. 4b) were both similar between subtypes (based on pairwise comparisons using the Kruskal Wallis test).

Comparison analyses and phylogenetic analyses of genes

To explore the differences between BVDV BJ–2013/BJ–2016 and other BVDV isolates, comparison analyses were performed based on nucleotide and amino acid sequences. The nucleotide identity of sequenced genomes was 80% when compared to each other. BVDV BJ–2013 showed highest nucleotide identity with BVDV 08GB44–1 (JQ418633.1) (99.9%) which isolated from South Korea. BVDV BJ–2016 shared highest nucleotide identity (96.4%) with BVDV HJ–1 from China. The amino acid similarity analysis of the two isolates showed the BVDV BJ–2013 shared highest amino acid similarity BVDV 08GB44–1 and BVDV BJ–2016 showed highest amino acid identity with BVDV HJ–01. Interestingly, there were 12 amino acid (EGNWLVNADRLM) insertion in non-structural protein 2 (NS2) of BVDV BJ–2013 compared with BVDV BJ–2016 (Fig. 5). It was similar with the insertion observed in other BVDV strains of cytopathogenic biotype BVDV, such as VE138cp05(LT907991), CP1741-A(AF220247), NADL(AJ133738), Vedevac(AJ585412).

The maximum likelihood phylogenetic trees (Fig. S3-Fig. S8) inferred from nucleotide sequences of genes N^{pro}, E^{ns}, E2, NS2, NS3, NS5B showed that isolate BVDV BJ–2013 was closely related with BVDV 08GB44–1 (South Korea) within the clade composed of Pestivirus A strains of genotype 1a. Isolate BVDV BJ–2016 was closely related to BVDV 08GB45–2 (South Korea), PJ(USA), Powder(USA), HJ–1 (China). These results were consistent with that of phylogenetic analysis based on polyprotein nucleotide sequence.

Discussion

Bovine viral diarrhea virus is an important pathogen of cattle worldwide, causing a variety of different symptoms and clinical diseases which can lead to major economic losses. In recent years, epidemiological studies have suggested that BVDV has become infectious to a growing number of animal species beyond cattle, including pigs and ruminants such as sheep, goats, yak, buffalo, llama,

alpaca, camel and deer[4, 21, 22]. The growing number of hosts that BVDV is capable of infecting presents a major challenge to BVDV eradication efforts.

In China, a variety of BVDV genotypes have been reported, including 1a, 1b, 1c, 1d, 1m, 1o, 1p, 1q, 1u, 2a and 2b[10, 11, 13, 23, 24, 25]) Among these genotypes, BVDV 1a and 1m predominate in China[4]. Epidemiological studies have shown that BVDV is endemic among many farms in China and that BVDV-infection is a serious problem across populations of farmed cattle. In China, to date, BVDV has been detected in cattle, pigs, sheep, deer, yak, goats and camels and it has been suggested that the host range is continuing to increase[10, 11, 12, 14, 23]. This may in part be related to improvements in the availability and use of BVDV detection technology and the growing number of animal species that are monitored for BVDV infection.

In the present study, we identified and characterized two BVDV strains: BVDV BJ-2013 and BVDV BJ-2016, from serum samples collected from cattle in Shandong province. We found that BVDV BJ-2013 and BVDV BJ-2016 were the member of Pestivirus A and belong to genotype 1a and 1b respectively. Phylogenetic analyses based on nucleotide sequences of polyprotein and genes of N^{pro}, E^{ns}, E2, NS2, NS3, NS5B and evolutionary analysis revealed that BVDV strains isolated from different regions showed close evolutionary relationships. It also reminded us again that the risk of trans-regional transmission of BVDV were what we must not neglect. The complex epidemic situation will bring new challenges to the eradication of bovine viral diarrhea (BVD).

It is well known that members of the *Pestivirus* genus are genetically highly heterogeneous[4]. Mutation and recombination are the two major factors by which genetic changes in BVDV occur. In the present study, we detected one recombinant BVDV strain: GS5 strain (KJ541471), which was closely related to strain KP941584 originating from the US and strain BVDV BJ-2016 isolated in this study. Bayesian coalescent analysis was performed to estimate the evolutionary rates in each codon of the polyprotein region of the BVDV genomes and revealed similar relative rates and dN:dS ratios in both BVDV genotypes (1a and 1b). It is suggested that the high inherent genetic variability of BVDV contributes to its adaptive ability to escape the host immune response, leading to the failure of immunity.

The expression of nonstructural protein NS3 was recognized as a specific marker for CP BVDV[26, 27]. The generation of CP virus can be induced by RNA recombination and introduction of a set of point mutation in NS2 gene[26]. In our study, 12 amino acids insertion were also found in NS2 of BVDV BJ-2013 and it was different with other CP BVDV strains. BVDV Oregon is a CP BVDV isolates. Amino acid residue S at position 1555 of the polyprotein play an important role for cleavage of NS2-3[20]. However, the amino acid residue at the same position of BVDV BJ-2013 and BVDV BJ-2016 were F. These suggested that CP virus BVDV BJ-2013 was generated from the insertion of these 12 amino acid insertion.

Conclusion

The present study has successfully isolated and identified two bovine viral diarrhea virus isolates (BVDV BJ–2013 and BVDV BJ–2016), from bovine serum. We have further conducted systematic analyses of the evolutionary phylogenetic relationships, which will inform our understanding of current and epidemics of BVDV infection and will provide a reference point for the implementation of BVDV prevention and control programmes in China.

Methods

Samples and cells

Two serum samples has been detected as BVDV positive by RT-PCR and stored at -80°C in our lab. One serum samples were collected, in 2013, from cattle at pasture in the province of Shandong, China. Collection of serum sample from cattle was performed under the permission of the owner and by workers of the pasture. Another serum samples was collected from commercial fetal bovine serum for cell culture from a Chinese manufacturer in 2016. Madin-Darby bovine kidney (MDBK) cells were Preserved by our laboratory. MDBK cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 8% fetal bovine serum (Gibco) and maintained at 37°C in an atmosphere with 5% CO_2 .

Virus isolation and identification

Virus isolation was performed on MDBK cells. Serum samples filtrated through $0.22\mu\text{m}$ filter were inoculated into monolayer culture of MDBK cells and maintained at 37°C for 1h. Afterwards, cells were washed thrice with phosphate buffered saline (PBS), and the culture medium was replaced with DMEM. The cells were observed for 5 days for the presence or absences of cytopathogenic effects by microscopy. The cultures were then freeze-thawed three times at -70°C and centrifuged at 8000 g for 5 min. The resulting supernatants were collected and used for sub-culturing.

Virus identification was performed by RT-PCR, indirect immunofluorescence assays (IFA) and electron microscopy techniques (EM). Primers used for BVDV identification were as follows: BVDV–5'UTRF(5'-TAgCCATgCCCTTAgtAggAC–3') and BVDV–5'UTRR(5'-ACTCCATgTgCCATgTACAgC–3'). For IFA, MDBK cells cultured in 48 well cell culture cluster were inoculated with polyclonal sera for 45 min at 24 h post-inoculated with the virus. Subsequently, the cluster was washed three times with PBS and then inoculated with fluorescein (FITC) labelled goat anti-mouse IgG antibody for 1 h at 37°C within dark place. Finally, the wells were sealed and examined by fluorescence microscopy. For examination under electron microscopy (EM), viruses collected after three blind passages were used. The supernatants were centrifuged at 12000 rpm for 30 min to remove cell debris, after which the resulting supernatants were re-centrifuged at 35000 rpm for 2 h. The precipitate was then resuspended in TNE buffer (PH 7.4), and negatively stained with 2% phosphotungstic acid. The specimens were examined with a transmission electron microscope (Hitachi, Japan).

PCR amplification and sequencing

The MDBK cells inoculated with the third passaged viral cell culture were centrifuged at $12,000 \times g$ at 4°C for 10 min in order to remove cell debris. Extraction of RNA from the resulting supernatants was performed by using the Viral RNA Extraction Kit (Aidlab Biotechnologies Co., Ltd, China). Single-stranded cDNAs were generated using M-MLV reverse transcriptase (Takara Biomedical Technology (Beijing) Co., Ltd, China) in accordance with the manufacturer's instructions. Subsequently, PCR was performed under conditions of 94°C for 3 min, followed by 35 cycles of DNA amplification according to the following protocol: 30 s at 94°C , 30 s at 55°C and 90 s at 72°C , followed by 7 min incubation at 72°C . The PCR products were cloned into a pMD18-T vector and sequenced. The primers used for PCR amplification were designed with Primer Premier 5.0 software (Table S1).

Evolutionary Analyses

Initially, we used the BLAST software suite (blastn and blastx) to identify viral sequences through their similarity to annotated viral genomes of Pestivirus A in GenBank. Based on the top hits produced by the blastn searches, 48 near-full length genomes were selected for subsequent analyses. The identifications (Genbank number) of the selected genomes are: KF896608; KX857724; KU159365; KP941586; HQ174292; JN380080; HQ174293; AF091605; AF041040; KJ541471; KP941584; LT907992; M96751; DQ088995; KC757383; KT943518; KT951840; KT951841; KP941581; HQ174295; JN380083; HQ174296; KF835698; KF835697; KT355592; JN380088; KU756226; JQ418634; KF772785; KP941587; JX297517; KP941583; KP941590; KP941589; KR029825; KP941592; KC963967; JX297519; JX297521; JX297513; JX297516; JX297512; JX297514; JX297518; JX297520 and JX297515. Next, BVDV genomes were aligned using Clustal X software[15]. The HKY model and gamma distribution were selected and inputted in to jModeltest software according to the likelihood ratio test (LRT)[16]. The maximum likelihood trees were inferred and clade support values were calculated using the approximate likelihood ratio test (aLRT) by PhyML software[17]. The maximum likelihood trees were edited and visualized using FigTree version 1.4.3 [<http://tree.bio.ed.ac.uk/software/figtree/>].

Detection of recombination

To determine the extent of recombination among the BVDV sequences mentioned in evolutionary analyses, we used RDP version 4 software[18], which incorporates a number of different statistical methods. A detailed explanation of each of these methods is given in the RDP user's manual (<http://darwin.uvigo.es/rdp/rdp.html>), but in brief: 3Seq performs an exact nonparametric test to detect recombination in triplets of sequences; maximum 2 (MaxChi) uses variable and invariable sites to detect recombination in pairs of sequences. The method generates random sequence pairs whose significance level is evaluated by the proportion of simulated sequence pairs with maximum 2 values higher than the those of the raw data; maximum match 2 (Chimaera) is a modification of the maximum 2 method that

uses variable sequence sites to calculate maximum 2 match statistics; Program Geneconv[19] detects gene conversions (recombination) by evaluating conserved substitutions in fragments between two sequences. Although evolutionary methods are not explicitly implemented in Geneconv, it is robust and has low levels of false positive detection of recombination, including those that arise due to rate heterogeneity and natural selection. Initially, we used default parameters. These were subsequently optimized to avoid detection of false positive recombination. In addition, we used window sizes of 20 to 50 as well as Bonferroni correction using *P*-values of 0.05 and 0.001.

Bayesian coalescent analysis

We used a Bayesian Markov Chain Monte Carlo (BMCMC) coalescent framework to infer the maximum likelihood trees. The BMCMC method incorporates the uncertainty of the measurements by considering the intrinsic errors in the tree reconstruction. The uncertainty in the estimated parameter is estimated by calculating the highest posterior density (HPD) interval, which contains 95% of the marginal posterior distribution of the data. After sufficient Markov Chain runs had been performed, which was indicated when the effective sample size (ESS) was higher than 100 and the parameters became stable, the mean and 95% HPD intervals of each parameter could be calculated. The Hasegawa-Kishino-Yano (HKY) model and a gamma correction were applied to all analyses; the evolutionary and demographic parameters were iteratively adjusted.

The Bayesian skyline plot method (BSP) was used to describe the dynamics of BVDV. It provides an unbiased description of genetic diversity over time (expressed as the product of the effective sample size (N_e) and generation time (τ)) because BSP contemplates a wide variety of demographic scenarios without formally assuming any determined model. The BSP model was found to be the best-fit model when compared with the constant coalescent model. Markov chain Monte Carlo (MCMC) processes were run for 3×10^8 generations with the initial 10 % of each run discarded as burn-in. The convergence of chains was evaluated using TRACER software, version 1.6.0 (available at: <http://beast.bio.ed.ac.uk/>). Runs were accepted when all parameters presented an effective sample size number (ESS) of greater than 200. The above analysis were performed using the Beast V 1.8.4 package[20].

Comparison and phylogenetic analyses of genes

To understand the difference between BVDV BJ–2013, BVDV BJ–2016 and other isolates, comparison analysis were performed with MEGA 6.0. The phylogenetic tree based on nucleotide sequences of genes (N^{pro} , E^{ns} , E2, NS2, NS3, NS5B) were constructed by MEGA 6.0 software using maximum likelihood method under the best model with 1000 bootstrap replicates. Available nucleotide sequences of genes N^{pro} , E^{ns} , E2, NS2, NS3, NS5B were collected from GenBank.

Abbreviations

BVDV: bovine viral diarrhea virus; CP: cytopathogenic biotype; CPE: cytopathic effects; DMEM: Dulbecco's modified eagle medium; FBS: fetal bovine serum; IFA: indirect immunofluorescence assay; MDBK: Madin-Darby bovine kidney cells; NCP: non-cytopathogenic biotype;

Declarations

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Authors' contributions

Conceived and designed the experiments: SJC and YMZ; Funding acquisition: SJC; Performed the experiments: CL, LL; Analyzed the data: CL, FV and É L; Wrote the paper: CL, FV, ÉL. All authors read and agreed to final version of manuscript.

Ethics approval and Consent to Participate

All experiments involving animals were conducted under protocols approved by Science Research Department (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences and conducted in compliance with the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. In this study, collection of serum sample from cattle was performed under the permission of the owner and by workers of the pasture. Any other animal experiment was involved in present study.

Consent for publication

Not applicable as no individual personal data were collected

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Figures

Figure 1

Identification of Pestivirus A by immunofluorescence assays (IFA) A: MDBK cells infected with isolate BVDV BJ-2013; D: MDBK cells infected with isolate BVDV BJ-2016; B and C: Untreated MDBK cells (control).

Figure 2

Phylogenetic tree constructed using near-full genomes of Pestivirus A. The phylogenetic tree was inferred using a maximum likelihood (ML) approach, assuming the data fitted an HKY model with discrete gamma correction and the rate of invariable sites. Values above branches indicate the branch support and were obtained from application of the approximate likelihood ratio test (aLRT) where a score of 1 is the highest score. The isolated BVDV strains are indicated by arrows in the phylogenetic tree.

Figure 3

Recombination pattern of Pestivirus A strain KJ541471 a: The phylogenetic tree was inferred using the concatenated fragment composed by nucleotides 1 to 6980 plus nucleotides 9567 to 11682 (indicated by the grey rectangle). b: The location of recombination breakpoints in the polyprotein region of isolate KJ541471, based on analysis using the MaxChi method, implemented in RDP software version 4. The recombinant isolate KJ541471 is indicated by a black arrow and the parental sequences by a black dot.

c: The phylogenetic tree was inferred using the fragment corresponding to nucleotides 6981 to 9566. Numbers above the branches are statistical support based on the approximate likelihood ratio test (aLRT).

Figure 4

Codon based evolutionary rates of the Pestivirus A polyprotein. a) The Fig. shows the relative amino acid rates in each codon of the polyprotein of the Pestivirus A estimated using the WAG model. Estimated evolutionary rates obtained from isolates of genotype 1A are shown as grey squares, and evolutionary rates obtained from isolates of genotype 1B are depicted as black dots in the. b) A codon based maximum likelihood method which was phylogenetically aware was used to estimate the number of

nonsynonymous (dN) and synonymous (dS) substitution rates in each codon site of the polyprotein. For simplicity, the lower panel shows only dN-dS values above 1 in each codon of the polyprotein. Estimated dN-dS values of subtype 1A are depicted by grey circles and values estimated for subtype 1B are depicted by black triangles. The y-axis depicts the rates and the x-axis indicates the codon positions in the Pestivirus A polyprotein. These rates were estimated using Mega software version 7.0.

Figure 5

Comparison analysis of NS2. Insertion of 12 amino acids were found in NS2 and the insertion sequence was different with other CP virus, including VE 138cp 05, CP 1741-A, NADL, and Oregon.

Supplementary Files

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