Genetic characterization of bovine viral diarrhoea virus detected in persistently infected cattle in Southern Minas Gerais, Brazil

Caracterização genética do vírus da diarreia bovina detectada em bovinos do Sul de Minas Gerais, Brasil, persistentemente infectados

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Abstract

Isolates of bovine viral diarrhoea virus (BVDV) detected in serum samples of two persistently infected animals (PI) identified in a herd located in the southern state of Minas Gerais, Brazil, underwent genetic characterization trough partial nucleotide sequencing and analysis of the 5' Untranslated Region (5'UTR) of the viral genome. The isolates were characterized as belonging to genotype BVDV-1, subgenotype BVDV-1b. The results of this study suggest BVDV-1b as an agent of importance in the occurrence of bovine viral diarrhoea (BVD) in the herds of the region. Moreover, the genotypic characterization of isolates of BVDV helps to better understand the epidemiology of the disease, as the genetic variability of BVDV interferes in the serological tests and has implications for the use of vaccines, whose majority is produced only with reference strains of BVDV. Therefore, the investigation on the genetic diversity of BVDV existing in Brazil is required for the improvement of the disease prevention and control measures.

Keywords: Bovine viral diarrhoea virus. Persistently infected animal. Genotypic characterization. Brazil.

Resumo

Isolados do vírus da diarreia viral bovina (BVDV) detectados em amostras de soro sanguíneo de dois animais persistentemente infectados (PI), identificados num rebanho bovino localizado na região sul do Estado de Minas Gerais, Brasil, foram submetidos à caracterização genética através do sequenciamento parcial de nucleotídeos da região 5'UTR do genoma viral. Os isolados foram caracterizados como pertencentes ao genótipo BVDV-1, subgenótipo BVDV-1b. Os resultados do presente estudo sugerem o BVDV-1b como um agente de importância na ocorrência da diarreia viral bovina (BVD) nos rebanhos da região. Ademais, a caracterização genotípica dos isolados do BVDV contribui para a melhor compreensão da epidemiologia da enfermidade, pois a variabilidade genética do BVDV interfere nos testes sorológicos e também possui implicações na utilização de vacinas, cuja maioria é produzida apenas com estirpes de referência do BVDV, requerendo, portanto, investigações sobre a diversidade genética do BVDV existente no Brasil para o aprimoramento das medidas de prevenção e controle da enfermidade no país.

Palavras-chave: Vírus da diarreia viral bovina. Animal persistentemente infectado. Caracterização genotípica. Brasil.

Introduction

Bovine viral diarrhoea virus (BVDV), accounted as a pathogen that has "many faces", has been related to a complex of syndromes that affects the reproductive, respiratory, digestive, circulatory, immune, lymphatic, musculoskeletal systems and central nervous system[1]. BVDV is a single-stranded RNA virus, whose variations in nucleotide sequence at the 5' Untranslated

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Region (5'UTR) of the genome allowed the classification into two genotypes, BVDV-1 and BVDV-2[2]. They are next to Border disease virus (BDV) and Classical Swine Fever Virus (CSFV) in four species of the genus *Pestivirus* belonging to the family *Flaviviridae*[3].

BVDV-1 and BVDV-2 strains can also present themselves in two different biotypes, cytopathogenic (CP) and non-cytopathogenic (NCP), being the latter the most predominant in nature[4]. Infection of the bovine fetus with the NCP biotype of BVDV before the development of immune competence (until 125 days of gestation) may result after birth in an animal that has persistent infection by BVDV throughout his life. The generation of persistently infected animals (PI) is more frequent when fetal infection occurs between 30 and 90 days of gestation, which can be established by both BVDV-1 and the BVDV-2[5]. The PI animal is considered to be the main source of BVDV infection because it eliminates a large amount of virus in secretions and excretions during all its life[6].

Many PI animals can be clinically healthy, although their life expectancy is low, since all have the risk of developing mucosal disease (MD)[7]. The MD is a fatal syndrome, caused by both BVDV-1 and BVDV-2, and is considered a sporadic form of BVDV infection[8]. Occurs when PI animals with NCP BVDV were infected with the homologous CP BVDV and, in most cases, the CP virus originates from NCP persistent virus through genetic recombination[9].

The genetic diversity that occurs among isolates of BVDV isolated in the field work, existent in nature as *quasispecies*, is a common characteristic to BVDV[9]. The isolates are characterized by great genetic, antigenic and pathogenic variation, as manifested by differences between the phenotype (virulence) and genotype. Such diversity has consequences on the disease clinical manifestations, laboratory diagnostic tests results and vaccines usage[4,10,11].

The occurrence of point mutations in the RNA virus is so common that, in general, it is estimated one mutation per 10,000 virus replication. Because the BVDV genome has approximately 12,300 base pairs, it is likely to occur at least one nucleotide change at each cycle of viral replication. Thus, each offspring is different from the original virus in one or more bases and a large number of mutants are created in every cycle of virus replication[9,10]. The accumulation of point mutations over time resulted in the segregation of BVDV into two genetically distinct genotypes then known as BVDV-1 and BVDV-2[12].

BVDV genetic groups from different parts of the world are defined from various regions of the genome[13]. Phylogenetic analysis demonstrated the existence of subgenotypes BVDV-1 and BVDV-2, 12 for genotype BVDV-1 (BVDV-1a, BVDV-1b, BVDV-1c, BVDV-1d, BVDV-1e, BVDV-1f, BVDV-1g, BVDV-1h, BVDV-1i, BVDV-1j, BVDV-1k, BVDV-1l)[4,14], and two for genotype BVDV-2 (BVDV-2a, BVDV-2b)[4,15]. An atypical pestivirus isolated in South America and Southeast Asia was identified (HoBi –like virus) and grouped with BVDV based on phylogenetic analysis, suggesting this group of viruses as BVDV-3[4].

In Brazil, Flores et al.[16] identified four isolates belonging to BVDV-1a, nine isolates to BVDV-1b and four isolates to BVDV-2. In the phylogenetic analysis performed by Vilcek et al.[17] with Brazilian BVDV isolates, four isolates were identified belonging to subgenotype BVDV-1a, two to BVDV-1b, one to BVDV-1d, one to BVDV-1k and three to BVDV-2. From 19 strains isolated in Brazil, Cortez et al.[18] found eight belonging to subgenotype BVDV-1a, three to BVDV-1b, two to BVDV-2a, four to BVDV-2b and two other were classified in a third group of samples termed as atypical. Lunardi et al.[19] identified, in the northern region of Parana State, a isolate belonging to BVDV-1b from an animal with the clinical form of bovine viral diarrhoea (BVD).

The high frequency of mutation found among BVDV isolates, the tendency for genetic recombination and the selective pressure imposed by host immune response lead to the genetic diversity of the virus[9]. As BVDV has a wide heterogeneity among the isolates, the objective of this study was to genetically characterize two BVDV isolates detected in two PI bovines in a herd located in the southern of Minas Gerais state to establish phylogenetic association with other strains already identified based on genomic sequences of the 5'UTR region.

Materials and Method

Samples. In 2006, two PI calves were diagnosed in the herd located in the town of Poço Fundo, southern Minas Gerais, by detecting the virus in paired samples of blood serum obtained in 3 and 4 weeks interval[20,21]. This herd, a typical example of the brazilian cattle herd, consisted of 76 crossbred beef cattle from different origins under extensive breeding management, and all animals are part of the same large lot. The serological analysis of herd, by virus neutralization test, showed that more than 90% of the animals were reactive to BVDV. The owner of the herd did not report the occurrence of clinical disease or reproductive disorders in the cattle.

The PI calves, one was a heifer and the other a calf, were between 4 and 5 months old when diagnosed, were healthy and showed no differences in development of other animals of the same age. However, the heifer, three months after performed the last harvest, showed clinical signs of incoordination and progressive weakness followed by death. Another calf had normal development, but according to the owner, was always weaker than the other animals of the same age.

Virus detection. The protocol described by Pilz, Alfieri and Alfieri[22], with some modifications, was used in search for BVDV to perform the polymerase chain reaction preceded by reverse transcription (RT-PCR). The RNA extraction was performed following the method proposed by Boom et al.[23], by using silica particles and guanidine isothiocyanate for nucleic acids purification. RT-PCR was performed using sense primers 103 (5' TAG CCA TGC CCT TAG TAG GAC 3' - genomic position 103-124) and 372 antisense (5' ACT CCA TGT GCC ATG TAC AGC 3' - genomic position 372-392), which amplifies a product of 290 base pairs (bp) and shares maximum similarity between BVDV-1 and BVDV-2[24].

Molecular characterization. For characterization on genotype and subgenotype, PCR samples were directly subjected to partial nucleotide sequencing of the 5'UTR region. The purification of PCR products was performed using the GXF PCR DNA kit and Gel Band Purification (GE Healthcare). The quantification was performed in agarose gel 2%, using the Low DNA Ladder marker Invitrogen™. The sequencing reaction was performed using the DNA DYEnamic ET Dye Terminator kit (GE Healthcare) in an automatic sequencer MegaBACE 1000 (GE Healthcare). Each PCR product was sequenced using primers sense 103 and antisense 372[24], aiming at consensus-sequence.

The quality index of chromatograms was checked with PHRED software[25]. After this verification, the chromatograms were analyzed with BioEdit software suite[26] for determining the consensus-sequence and the identity was confirmed by BLAST software[27]. Alignment of sequences was performed using CLUSTAL W software, included in the BioEdit suite[26], and the similarity values between sequences were obtained from MATGAT software[28]. The MODELTEST software[29] was used to determine the best evolutionary model of nucleotide substitution in the group of analyzed sequences.

The phylogenetic reconstruction was performed in the software MEGA version 4.0[30], using as algorithm the Neighbor-joining method (Kimura 2 parameter) and the bootstrap values were calculated using 1000 pseudo-replicates. In this reconstruction, there

were used for comparison the sequences recorded in the GenBank[31] of reference strains of BVDV-1 and BVDV-2 as well as the strains of each subgenotype BVDV isolated in Brazil, besides strains of Border disease virus (BDV) and classical swine fever virus (CSFV) used as outgroups.

The sequences identification used for comparison, the GenBank accession number and origin of each isolate were, respectively: NADL - M31182 (USA); Singer - DQ088995 (USA); BR275 - U94915 (Brazil); R1935/72 - U94916 (Brazil); Nose - AB019670 (Japan); Oregon C24V - AF091605 (England); BR-UFMG-4 - AF258613 (Brazil); Osloss - M96687 (Germany); NY-1 - L32879 (USA); 3P - AF244968 (Argentina); UEL1-BR/04 - EF406123 (Brazil); 1248/01 - AY159545 (Spain); M079B-91 - U97410 (Mozambique); 2900/83 - AJ304375 (Germany); 3186V6 - AF298062 (Italy); 4998/89 - AJ304385 (Germany); A - AF298064 (Slovakia); G - AF298066 (Slovakia); 23-15 - AF298059 (England); 890 - U18059 (USA); Soldan - U94914 (Brazil); LV-86 - AF410787 (Brazil); VS-123 - AF410790 (Brazil); BDV/Rudolph - AB122086 (Japan); CSFV/MP - AY182247 (India).

Results

The nucleotide sequences of the BVDV isolates detected in the two PI animals showed differences in some bases. Thus, they were considered distinct and named BR-UNESP-JAB 1 and BR-UNESP-JAB 2. These sequences were deposited in GenBank under access numbers FJ895327 and FJ895328, respectively. In the phylogenetic analysis, the isolates were characterized as belonging to genotype BVDV-1, subgenotype BVDV-1b (Figure 1), sharing highest similarity (91.7% and 92.3%) with the representative subgenotype BVDV-1b isolate Osloss[32] (Table 1).

BVDV variant isolates are grouped based on the homology of the viral genome segments sequences. The genotypes show 60% of similarity in their base sequence, the subgenotypes within genotypes have between 80% and 85% of similarity and viral strains in the group of subgenotypes represent the group with similarity exceeding 90%[9]. The isolated strains were characterized in this latter condition, since their genetic similarity was higher than 90% when compared with strains of BVDV-1b (Table 1).

Discussion

The detection of BVDV-1b in this study complements the heterogeneity shown by the isolation and genetic characterization of BVDV conducted in Brazil, with the detection of BVDV-1a, BVDV-1b and BVDV-2[16]; BVDV-2a and BVDV-2b[15]; BVDV-1a, BVDV-1b, BVDV-1d and BVDV-2[17]; BVDV-1a, BVDV-1b, BVDV-2a and BVDV-2b[18]; and BVDV-1b[19]. However, from the isolates of BVDV isolated by Flores et al.[16], BVDV-1b was the most frequent subgenotype. In the U.S., BVDV-1b was also more frequent among the isolates[33,34,35,36], as well as in the phylogenetic analysis of 25 BVDV isolates in Peru and Chile, 23 of which belonged to subgenotype BVDV-1b[37].

BVDV-1b isolates characterized in this study were derived from PI calves. In the study by Fulton et al.[35], BVDV-1b was the most commonly detected subgenotype in PI calves. In a study performed by Everman and Ridpath[38], BVDV-1 was closely associated to persistent infections, the occurrence of congenital defects and birth of weak calves. On the other hand, BVDV-2 was found mainly in cases of abortions and in cases of animals with clinical signs of BVD[11]. However, the BVDV isolated by Lunardi et al.[19] in a bovine with symptoms of hemorrhagic BVD belonged to BVDV-1b subgenotype. The BVDV-1b predominance in the diagnosis of PI animals among herds could be due to the absence of such subgenotype strains in the majority of available vaccines[33,34] or the animals that have

the BVDV-1b persistent infection are likely to survive longer and hence the greater the spread of the virus[39].

The replication of BVDV in PI animals occurs in high quantities for months or even years[40]. However, this abundant viral proliferation does not seem to generate a genetic diversity of the virus, possibly because these variants would be eliminated by PI animals immune system[10,40]. Some studies have shown that PI animals stabilize BVDV strains, making them specific to each herd[41]. The emergence of mutant BVDV would result from the high frequency of mutations or genetic recombinations undergone by the virus in acute infections of non PI animals[9,10]. In MD, the CP virus originates from NCP persistent virus through genetic recombination[9] and the expression of cytopathogenicity results from cleavage of non-structural protein NS2-3 of the NCP BVDV[42].

Results found in several studies paid attention to the issue of geographical distribution of BVDV genotypes and subgenotypes in certain regions and even countries, as the BVDV strains were geographically restricted, including with predominance of a particular subgenotype in a region[13,43,44,45,46]. As the isolates of BVDV-1b were detected in a herd located in southern Minas Gerais state, it is suggested that isolates of the same subgenotype could be present in other infected herds in the same region.

BVDV-1b predominates in respiratory diseases[11,34,47]. Dairy production is characteristic of the southern Minas Gerais state and its herds are raised in more intensively, which contributes to a higher incidence of respiratory disease, not only in calves, but also in adult animals. So, the BVDV infections in cattle herds in this region should not only be related to changes in the reproductive sphere, but also to the occurrence of respiratory diseases. The involvement of the virus is not only limited to its role as immunosuppressive agents, which favors the installation of other pathogens, but also act as a primary infectious agent, playing an important role in bovine respiratory complex[12,35]. However, in the herd where was diagnosed PI animals, was not reported the occurrence of respiratory diseases.

The genetic variability of BVDV interferes on laboratory diagnostic tests, as it reflects in the antigenic diversity and commits the results obtained by serological tests[10]. Genetic diversity also influences when using vaccines containing different subgenotypes from those circulating in cattle herds[4,34], since most are produced only with reference strains of BVDV-1a[32]. According to Fulton et al.[33], vaccines containing only BVDV-1a induced lower neutralizing antibody titers against BVDV-1b strains. In Brazil, antigenic analyses of national isolates of BVDV and tests of commercial vaccines have shown the necessity of reformulation of BVDV immunogens used in vaccines[15,48,49].

The existence of subgenotypes is a reflection of the BVDV diversity that exists in nature[34]. This diversity found throughout the world continues to expand, as new populations are being surveyed and a growing number of genome sequences is being characterized[50]. The characterization of the circulating genotypes in cattle from a particular region can contribute to a better understanding of the epidemiology and pathogenesis of BVDV infections, and influence the choice of strains to be used on vaccination[51]. Thereby, the knowledge of the genetic diversity of BVDV found in Brazil requires studies to improve the prevention and control measures of BVD in the country.

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