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Genetic typing of bovine viral diarrhea virus isolates from Costa Rica

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ABSTRACT

Sixteen bovine viral diarrhea virus (BVDV) isolates collected in Costa Rica between 1987 and 2006 from dairy cattle were analyzed by RT-PCR and determined to belong to BVDV species 1. Furthermore, eleven of these isolates were genotyped using the nucleotide sequences of the Npro region of the viral genome. Phylogenetic analysis indicated that all samples examined clustered within the BVDV-1b subtype.

KEYWORDS: bovine viral diarrhea virus, BVDV-1b, genetic typing, phylogenetic tree, Costa Rica.

Genotipificación de aislamientos del virus de la diarrea viral bovina en Costa Rica

RESUMEN

Dieciséis aislamientos del virus de la diarrea viral bovina (BVDV) recolectados entre 1987 y 2006 de hatos lecheros de Costa Rica fueron analizados mediante la técnica de RT-PCR y determinados como pertenecientes al genotipo 1. Once de estos aislamientos fueron genotipificados utilizando la secuencia de nucleótidos de Npro del genoma viral. El análisis filogenético determinó un único genogrupo en estos aislamientos, BVDV-1b.

PALABRAS CLAVES: Virus de la diarrea viral bovina, BVDV-1b, genotipificación, árbol filogenético, Costa Rica.

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) has been classified within the genus *Pestivirus* of the family *Flaviviridae* (Wengler et al., 1995). It has a positive single-stranded RNA genome of approximately 12.3kb size, encoding one open reading frame (ORF) that is translated into a single polyprotein of about 4000 kDa (Collett et al*.,* 1988; Meyers and Thiel, 1996). The polyprotein is subsequently cleaved into 4 structural and 6 to 7 non-structural proteins by viral and cellular proteases (Thiel et al., 1993). The ORF, which starts with the N^{pro} viral autoprotease, is flanked at the 5' and 3' termini by untranslated regions (5'-UTR, 3'-UTR) (Collett et al., 1988; Meyers and Thiel, 1996).

Two species of BVDV, BVDV-1 and BVDV-2, have been described (Pellerin et al., 1994; Ridpath et al., 1994). The genotype is determined by genetic typing, usually using sequences from the 5'-UTR, N^{pro} and E2 genetic regions. BVDV is genetically highly variable, particularly BVDV-1, where at least 11 genogroups have been identified (Vilcek et al., 2001). BVDV-1a and BVDV-1b seem to be the most prevalent genogroups worldwide (Fulton et al., 2003; Vilcek et al., 2004). In addition, due to their effects on permissive cells, two biotypes can be distinguished, the cytopathic (cp) and the non-cytopathic (ncp) (Donis and Dubovi, 1987; Greiser-Wilke et al., 1992).

Animals which become infected intrauterinely with ncp-BVDV (50 to 150 days of gestation) are epidemiologically important, as they may become tolerant to the virus. These persistently infected (PI) animals are the major source of virus maintenance and shedding in dairy farms and readily infect all susceptible animals. Many persistently infected animals appear clinically normal, while others may be weak at birth (Kelling, 1996).

BVDV-1 infections involve mainly respiratory, reproductive and enteric diseases, causing considerable economic losses to the cattle farming industry worldwide (Baker et al., 1954; Brownlie et al., 1987; Nettleton and Entican, 1995). BVDV-2 causes similar clinical signs to BVDV-1, except that infection with a highly virulent isolate may lead to thrombocytopaenia and fatal haemorrhagic syndrome (Corapi et al., 1989; Carman et al., 1998; Liebler-Tenorio et al., 2002).

Genetic and antigenic diversity of BVDV is important to consider for the design of effective vaccination programs (Fulton et al., 2003; Vilcek et al., 2004). The objective of this study was to determine genetic diversity of BVD viruses present in dairy herds from Costa Rica, Central America, and to describe management routines carried out in these farms that are important for the epidemiology of the disease.

MATERIALS AND METHODS

Virus Samples

A total of 16 BVDV isolates were analyzed in this study. Samples were collected in 2006 from dairy herds (Figure 1) or submitted to the Laboratory of Virology between 1987 and 2006. Seven positive animals were detected among the 1,980 serum samples collected and tested individually for BVDV antigen using a capture ELISA (HerdCheck, BVDV/Serum Plus, Idexx Laboratories, Österbybruk) during 2006 from 29 specialized dairy farms from Cartago, Heredia, Guanacaste, San José and Alajuela. The number of animals tested for BVDV antigen to determine the percentage of PI animals was determined based on Cannon and Roe (1982) (1% expected prevalence of PI

animals and a 95% confidence level), taking into account a total of 60,000 dairy cattle and 1,600 dairy herds in Costa Rica (Anonymous, 2000). Surveys were carried out on these farms to obtain information about cattle management practices (purchase of animals, BVDV examination and vaccination practices, replacement strategies), handling of calves (elimination strategies and location of calves on the farm) and observation of BVDV associated diseases (retarded growth, abortion, mucosal disease, hemorrhagic syndrome) (Table 2). The additional 9 positive samples were clinical submissions to the Laboratory of Virology, Universidad Nacional in Costa Rica, over the last 20 years (1987-2006). All 9 samples were tested the day of submission for virus isolation and tested afterwards under direct immunofluorescence. Non-infected Madin-Darby Bovine Kidney (MDBK) cells were included as negative control. The isolates were kept at –70°C, until molecular testing was carried out in 2006. Data concerning the year of submission and the region of origin was available only for 5 samples.

RNA Isolation

All 16 BVDV positive samples were subject to 4 passages on MDBK cell cultures grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% BVDV-free fetal bovine serum (FBS) and antibiotics (200 IU of penicillin/ml, 200 µg of streptomycin/ml). Total RNA was extracted from cell culture supernatants using QIAamp Viral

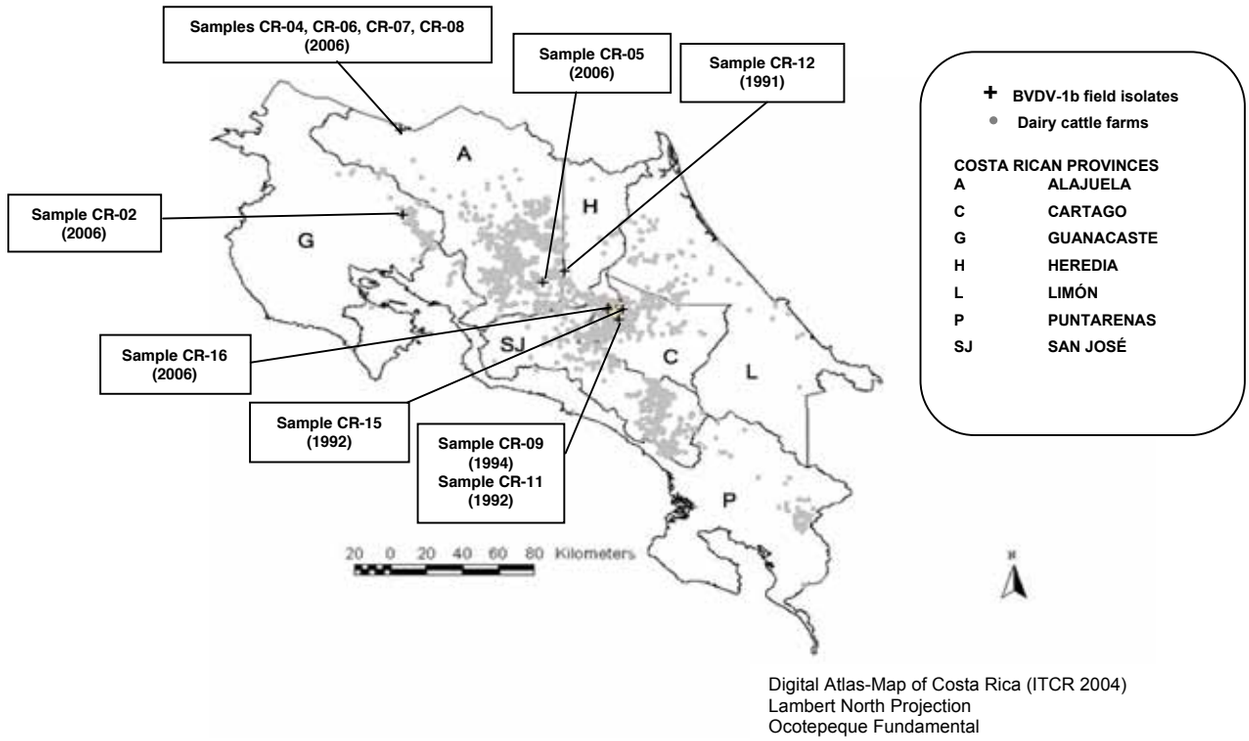

Figure 1. Geographical distribution of dairy cattle farms and the corresponding BVDV-1b field isolates in Costa Rica, indicating the year of isolation of the field strains (only samples with known geographical origin are shown)

RNA Mini Kit® (Qiagen, Hilden) following manufacturer's instructions. Non-inoculated MDBK cell cultures were included as negative controls.

Reverse Transcriptase PCR (RT-PCR) and Sequencing

Synthesis of cDNA was performed with random hexamers (Fermentas Inc, Glen Burnie, Maryland) in 40 μl total reaction volume. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out as previously described (Letellier et al*.,* 1999). A 221 bp DNA product was amplified from the 5'-UTR for BVDV-1 and BVDV-2. Primers used were B3 (5'-GGT AGC AAC AGT GGT GAG-3') and B4 (5'-GTA GCA ATA CAG TGG GCC-3') to determine BVDV-1 and primers B5 (5'-ACT AGC GGT AGC AGT GAG-3') and B6 (5'-CTA GCG GAA TAG CAG GTC-3') to determine BVDV-2, respectively. RT-PCR products were visualized by 2% agarose gel electrophoresis and subsequent staining in ethidium bromide. Using primer OL100 (5'-CAT GCC CTT AGT AGG ACT AGC-3') and primer 1400R (5'-ACC AGT TGC ACC AAC CAT G-3'), a 1341 bp product encompassing the Npro region was amplified (Becher *et al*., 1999). PCR amplicons were purified using QIAquick® PCR Purification Kit (Qiagen) and sequenced in both directions (MWG Biotech AG, Martinsried, Germany).

Phylogenetic Analysis

Nucleotide sequences were edited and analysed using the BioEdit software, version 7.0.9.0 (Hall, 1999). As with most BVDV isolates, only shorter fragments encompassing about 300 nu are available from GenBank. The corresponding fragments were used for calculation of the phylogenetic tree. Alignment and calculation of the unrooted phylogenetic neighbour-joining tree was performed in ClustalX 1.83 (Thompson et al., 1997) and bootstrapping was performed with 1,000 replicates. For the graphic output, Tree View software, version 1.6.6 (Page, 1996) was applied. The EMBL accession numbers of the sequences included as controls are listed in Table 1.

RESULTS

A total of 7 BVDV positive samples were detected in 4 out of 29 tested dairy farms in 2006, determining a percentage of 0.35% of PI animals. The survey yielded the following herd management practices: all farms had a veterinarian visit periodically (100%), 69% of the farms obtained their replacements completely within their own farm, whereas for the rest (31%) replacements were purchased from other farms. All the farms reported abortion and calves with retarded growth, and in 83% of the cases weak calves were eliminated from the farms. A total of 90% of the farms kept their calves at a short distance from adults, whereas 10% of the farms raised calves together with adult bovines. BVDV vaccines were used in 14% of the farms (Table 2).

The analysis by RT-PCR indicated that the 16 BVDV-positive samples for antigen capture ELISA and direct immunofluorescence assay belonged to BVDV species 1. BVDV-2 isolates were not detected. The determination of the nucleotide sequence of the Npro coding region was performed for 11 of this 16 BVDV field isolates. Nine field isolates (CR-01 to CR-06, CR11, CR-14 and CR-16) had 100% identical sequences in the fragment analyzed, whereas the other 2 field isolates (CR-12 and CR-15) had single base exchanges. The resulting phylogenetic tree indicated

Table 1. Accession numbers of the N^{pro} sequences and origin of the BVDV isolates representing the major BVDV-1 genetic subtypes used for calculation of the phylogenetic tree.

Isolate	Year of isolation	Country	Subtype	EMBL Acc. No.
NADL	-	US	BVDV-1a	AJ1333738
Singer	-	US	"	AY323892
221	-	-	"	AY735469
SD-1	-	US	"	M96751
Deer-NZ2	1980	NZ	"	U80904
Oregon C24V	-	US	"	AF091605
S6	-	-	"	AY735494
Osloss	-	DE	BVDV-1b	M96687
S14-2	-	-	"	AY735491
TGAN	-	US	"	AF145365
NY-1	-	US	"	AF145363
Asturias-31	1999	ES	"	AY182158
Asturias-36	1989	ES	"	AY182161
Girona-33	2000	ES	"	AY182159
P	1998	AT	"	AF287288
Buffalo-A1	1994	AU	BVDV-1c	U80901
Astur2-36	1989	ES	"	AY182162
Trangie-Y546	-	AU	"	AF049222
F	1998	AT	BVDV-1d	AF287284
22531	-	-	"	AY735472
SH9	1991	DE	"	AF1444473
S14-1	-	-	BVDV-1e	AY735490
20V661-1	1996	FR	"	AF298058
J	1998	AT	BVDV-1f	AF287286
W	1998	AT	"	AF287290
X-159-01	-	SI	"	AY323892
L	1998	AT	BVDV-1g	AF287287
Leon-6	2000	ES	BVDV-1h	AY182144
23/15-UK	1997	UK	BVDV-1j	AF287279
Deer-GB-1	1986	UK	Deer	U80902
Soldan	-	BR	BVDV-2	AY735495
1373	-	US	"	AF145967

AT, Austria; AU, Australia; BR, Brazil; DE, Germany; ES, Spain; FR, France; SI, Slovenia; UK, United Kingdom; US, United States

Table 2. Results of the survey carried out in 31 dairy farms of Costa Rica to determine cattle management practices, handling of calves and BVDV associated diseases (only affirmative answers were recorded).

Topics	#Herds(%)
Cattle management practices	
1. Are animals purchased from other farms?	9 (30.0)
2. Is BVDV testing performed before purchasing animals?	0 (0.0)
3. Is replacement made completely with own animals?	22 (70.0)
4. Does a veterinarian periodically visit the farm?	31 (100.0)
5. Are BVDV vaccines used in the farm?	4 (13.0)
Handling of calves	
1. Are weak or retarded calves culled?	26 (84.0)
2. Location of calves on the farm:	
a. Totally isolated from adult bovines	0 (0.0)
b. At a short distance from adult bovines	28 (90.0)
c. Together with adult bovines	3 (10.0)
BVDV associated diseases	
1. Have calves with retarded growth ever been recorded on the farm?	31 (100.0)
2. Have abortions ever been recorded on the farm?	31 (100.0)
3. Has mucosal disease ever been recorded on the farm?	0 (0.0)
4. Has hemorrhagic syndrome ever been recorded on the farm?	0 (0.0)
5. Has BVDV ever been diagnosed on the farm in association with abortions?	0 (0.0)

that the 11 field isolates from different geographic areas and collected at different dates belonged to the BVDV-1b subgroup (Figures 1 and 2). They formed a cluster that was separated by a high bootstrap value from other isolates in this subgroup.

DISCUSSION

In the present study, a low percentage of PI animals was detected, which could be consequence of good herd management strategies in most of the dairy farms surveyed. Another possible reason for a low percentage of PI animals could be the sampling method used, since approximately 25% of adult animals were tested in each farm. The PI animals detected in two farms with good herd management strategies were 2 heifers identified only by laboratory testing. The other two herds where 5 PI animals were detected had an open herd management system, did not eliminate weak or retarded calves and vaccinated against BVDV. The PI

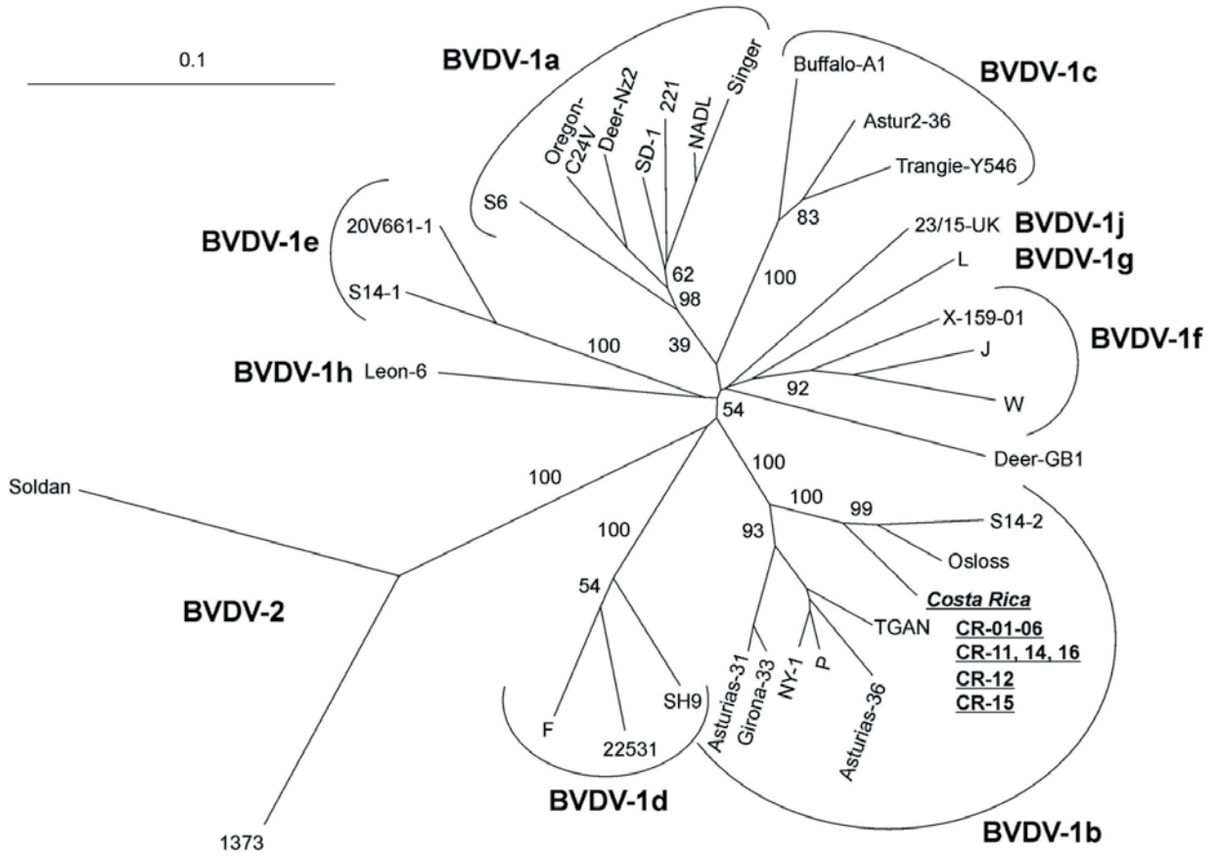

Figure 2. Unrooted Neighbor-Joining tree constructed from 330 nucleotides of the N^{pro} gene of the BVDV isolates from Costa Rica (bold, underlined). Bootstrapping was performed for 10.000 replicates and values are indicated as %. Bar: nucleotide substitutions per site.

animals detected in these two farms were 4 calves (5 to 9 months of age) showing reduced growth and 1 heifer (Greiser-Wilke et al., 2003).

The phylogenetic tree calculated using the 300 nu fragment of the Npro clearly allowed for distinguishing between the individual BVDV subgroups, showing high bootstrap values (Nei and Kumar, 2000).

The results of the present study indicate that BVDV field viruses from Costa Rica could be genetically homogeneous with minimal variability over time (from 1987 to 2006) and geographical origin. These results are in accordance with reports from the United States of America, where BVDV-1b was recognized as the most prevalent genotype of BVDV (Fulton et al., 2003; Tajima, 2006). In contrast, genotyping of BVDV isolates from South America revealed considerable genetic heterogeneity (Vilcek et al., 2004). The sanitary barrier to prevent the introduction of Foot and Mouth Disease into Costa Rica prohibits the import of cattle from South America. However, an increase of import of livestock from Canada, USA and Mexico to Costa Rica occurred mainly in the 60s. This might be the reason why Costa Rican isolates were homogenous and all in genogroup BVDV-1b (OIE, 2009).

CONCLUSIONS

The predominance of BVDV-1b in Costa Rica may have an impact on vaccination programmes. All vaccines used in Costa Rica in 2006, until today, contained BVDV-1a and BVDV-2a. These vaccines may not properly protect bovines against BVDV-1b, since only low neutralizing antibody titers against BVDV-1b were induced by BVDV-1a in commercial vaccines (Fulton et al., 2002; Fulton et al., 2003); however, a commercially VDVB-1b vaccine is not yet available (Ishmael, 2009). It will be interesting in future studies to determine the predominant clinical signs caused by this genogroup in susceptible bovine herds from Costa Rica, since BVDV-1b is generally isolated from calves with respiratory symptoms (Fulton et al,. 2002).

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