Molecular characterization of pestiviruses isolated from bovines in Chile

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Abstract

Thirty-three pestiviruses isolated from bovines on different farms in Chile were characterized at the molecular level. The 5′-untranslated region (5′UTR) of the isolates was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and subsequently digested with restriction enzymes (RE) *Bgl* I, *Xho* I and *Pst* I. Furthermore, the isolates were amplified by differential RT-PCR, which selectively amplified bovine viral diarrhea virus type 2 (BVDV-2) but not bovine viral diarrhea virus type 1 (BVDV-1). Of the 33 isolates, 16 were classified as BVDV-1 and 17 as BVDV-2. Phylogenetic analysis of the PCR-amplified fragments from 14 isolates showed the presence of viruses belonging to the BVDV-1a, BVDV-1b, BVDV-1c, and BVDV-2 types. Antigenicity of some viruses belonging to both genotypes was studied by cross-seroneutralization, revealing great antigenic differences among them. It is concluded that BVDV viruses circulating in Chile are genetically and antigenically heterogeneous, comprising isolates of genogroups 1 and 2.

Keywords: Pestiviruses; Bovine viral diarrhea virus; Genotyping

1. Introduction

Bovine viral diarrhea virus (BVDV), a small enveloped RNA virus, is a member of the genus *Pestivirus*, family *Flaviviridae*, which also includes Classical Swine Fever Virus (CSFV) and Border Disease Virus (BDV) (Meyers and Thiel, 1996; Nettleton et al., 1998). BVDV is present worldwide, and the broad spectrum of clinical syndromes that it produces make it one of the most important viral pathogens of cattle and responsible for significant economic losses for cattle producers throughout the world (Baker, 1987; Brownlie et al., 1987).

BVDV usually causes no or only mild clinical symptoms with a rapid recovery. However, infection of immunocompetent animals with BVDV may also cause clinical signs characterized by transient fever, leukopenia, diarrhea, respiratory disease, abortion,

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congenital defects in calves, reproductive failure and, through an immunosuppresive effect, contribute to the severity of other infectious diseases or disease complexes (Radostits and Littlejohns, 1988). Transplacental infection of the fetus before onset of immunological maturity, causes animals persistently infected with BVDV (PI), which are the principal source of infection to susceptible animals in the same herd. Superinfection of PI animals produces the fatal bovine viral diarrhea-mucosal disease (BVD-MD).

In the recent years, new hypervirulent BVDV strains have been associated with severe outbreaks, which are characterized by fatal acute diarrhea and fatal thrombocytopenia and have been reported to cause deaths in all age groups (Rebhun et al., 1989; Corapi et al., 1990; Pellerin et al., 1994). Genetic studies have demonstrated that the isolates associated with these outbreaks were different from the classical BVDV strains.

The pestivirus genome is a positive-stranded molecule of RNA, approximately 12.5 kb in length. The genome contains a single open reading frame flanked by a 5′ and a 3′ untranslated region (UTR) (Meyers and Thiel, 1996). The 5′UTR of almost 400 nucleotides is the most conserved part of the pestivirus genome and has allowed for the genetic typing of BVDV isolates into two genotypes, BVDV-1 and BVDV-2, with at least 11 genetic subgroups within genotype BVDV-1 (Pellerin et al., 1994; Ridpath et al., 1994; Vilcek et al., 2001).

The BVDV-1 genotype comprises the classical BVDV strains, including commonly used laboratory strains that are usually associated with mild clinical signs. The BVDV-2 genotype not only comprises BVDV strains associated with mild clinical signs as well, but also all the newly described BVDV strains associated with acute BVD/hemorrhagic disease outbreaks. BVDV-1 strains are distributed worldwide, but the BVDV-2 strains have been identified mainly in the USA and Canada, with a few reports of their presence in Europe, Japan and South America (Wolfmeyer et al., 1997; Nagai et al., 1998; Shimazaki et al., 1998; Flores et al., 2000; Jones et al., 2001; Couvreur et al., 2002; Vilcek et al., 2003).

BVDV infection is widely spread among Chilean cattle with a serological prevalence ranging from 59.7% to 69.2% in unvaccinated animals from different regions of the country (Celedón et al., 1996, 1997a). Up until now, BVDV has been isolated frequently from aborted fetuses and dead or persistently infected calves (Celedón et al., 1997b, 1998). In this paper we report the genetic and antigenic diversity of pestivirus isolates circulating in Chilean bovines.

2. Material and methods

2.1. Viral isolates

The 33 viruses included in this study were isolated from naturally infected cattle from different regions of Central Chile during the period 1993–2001. The isolates were obtained from organ suspensions of aborted fetuses, and buffy coat or serum from calves and adult cattle suffering enteric, respiratory or reproductive problems submitted for virus diagnosis at the Unit of Virology, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile. The viruses were propagated on monolayers of MDBK cells, determined to be free of BVDV, and grown in Eagle's Minimum Essential Medium supplemented with 10% horse serum. All infections were monitored by direct immunofluorescence (DIF) assay. DIF was performed in acetone-fixed cells, using a FITC-conjugated anti-BVDV policlonal serum (Central Veterinary Laboratory, Addlestone, Surrey, UK). The identification and origin of viruses, as well as predominant clinical syndromes present in the animal from which the viruses were isolated, are summarized in Table 1. All viruses are non-cytopathic.

2.2. Primers

Primers used for amplification of the 5′UTR region of the pestivirus genome have been described previously. The region corresponding to position 108–395 according to the NADL genome sequence was amplified using the panpestivirus-specific primers 324/326 (Vilcek et al., 1994). The product of in vitro amplification was a 288 bp DNA fragment. The region corresponding to position 191–315 according to the NADL genome sequence was amplified using the BVDV-2-specific primers JP1/JP2 (Ridpath et al., 1994). The product of in vitro amplification was a 117 bp DNA fragment.

Table 1
Characteristics and origin of the Chilean BVDV field isolates analyzed in this study

Origin[a]	Year of isolation	Clinical symptoms[b]	Genotype	Accession numbers
CH113F1	1995	PI	1b	AY671978
CH115F1	1995	Healthy	1	–
CH133F2	1995	RP	2	–
CH134F2	1995	PI	2	–
CH163F3	1995	PI	1	–
CH167F3	1995	Healthy	1	–
CH170F3	1995	PI	1b	AY671979
CH171F3	1995	Healthy	2	–
CH184F4	1995	RP, PI	1	–
CH193F5	1995	Healthy	1	–
CH226F6	1995	Healthy	1b	AY671980
CH327F7	1995	Healthy	1	–
CH375F7	1995	Healthy	2	–
CH379F7	1995	Healthy	2	–
CH419F7	1995	Healthy	2	–
CH470F8	1995	PI	2	AY671984
CH476F9	1995	Healthy	2	–
CH478F9	1995	Healthy	2	–
CH481F9	1995	Healthy	1b	AF356503
CH511F10	1993	AF	1c	AF356504
CH515F11	1996	Healthy	2	AY671985
CH565F13	1996	RP	1b	AY671981
CH578F13	1996	RP	2	–
CH615F14	1996	RP	1b	AY671982
CH618F14	1996	RP	1	–
CH649F15	1996	RP	2	AY671986
CH692F16	1996	RP	1c	AY671983
CH693F16	1996	Healthy	2	AF356505
CH735F17	1996	Healthy	2	–
CH750F18	1996	RP	2	–
CH804F19	1998	Healthy	2	–
CH809F19	1998	LD	2	AY671987
CH888F20	2001	DC	1a	AY671977

[a] Letters after case number indicate the farm (F) where the isolate was obtained.

[b] PI: persistent infection; RP: reproductive problems; AF: aborted foetus; LD: dead calf with lethal diarrhea; DC: dead calf, five days after birth.

2.3. RT-PCR

Total RNA was extracted from infected cells using Trizol LS (Life Technologies) according to the manufacturer's instructions. Synthesis of cDNA was performed in 20 μl final volume for 45 min at 55 °C under the following final conditions: 5 μl of heat-denatured RNA (2 min at 94 °C), 10 pmol of primer 326 or JP2, 50 mM Tris-acetate pH 8.4; 75 mM KCH_3COO; 0.5 mM dithiothreitol; 8 mM $Mg(CH_3COO)_2$; 0.5 mM of each dATP, dCTP, dGTP, dTTP; 2 U of RNase inhibitor (RNase OUT, Life Technologies) and 1 U of Thermoscript reverse transcriptase (Life Technologies). After reverse transcription, the reactions were heated to 90 °C for 5 min in order to inactivate the enzyme.

Amplification of cDNA by PCR was carried out in a total volume of 50 μl, under the following conditions: 5 μl cDNA, 25 mM Tris–HCl pH 8.9, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM dithiothreitol, 0.5 mM of each dATP, dCTP, dGTP, dTTP, 10 pmol of primers 324 and 326 or JP1 and JP2, and 2.5 U Taq DNA polymerase (Life Technologies). The thermal profile used consisted of 30 cycles of the following: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min. Amplified products were separated by electrophoresis in 8% PAGE gels run in 90 mM Tris, 90 mM boric acid, 2 mM EDTA at 18V/cm of gel for 45 min. DNA bands were visualized by the silver staining method (Espejo and Escanilla, 1993).

2.4. Genetic typing of the viral isolates with restriction enzymes

The viral isolates were typed with restriction enzymes as described by different authors (Vilcek et al., 1994; Harpin et al., 1995; Paton et al., 1995). Briefly, 2–4 μl of amplified products obtained after RT-PCR using the 324/326 primers were digested with 5 U of *Bgl* I, *Xho* I or *Pst* I, in 20 μl final volume for 4 h at 37 °C, according to the manufacturer's protocol (Life Technologies). The digestion products were separated by electrophoresis in 15% PAGE gels run in 90 mM Tris, 90 mM boric acid, 2 mM EDTA at 18 V/cm of gel for 90 min. DNA bands were visualized by the silver staining method (Espejo and Escanilla, 1993).

A BVDV-1 isolate correspond to viruses with amplified products not digested by *Bgl* I, but digested by *Xho* I (two DNA fragments of 117 and 171 bp) and *Pst* I (two DNA fragments of 233 and 55 bp) (*Bgl* I/*Xho* I/*Pst* I = −/+/+). A BVDV-2 isolate correspond to viruses with amplified products not digested by *Bgl* I and *Pst* I, but digested by *Xho* I (117 bp + 171 bp) (*Bgl* I/*Xho* I/*Pst* I = −/+/−). A BDV isolate correspond to viruses with amplified products not digested by *Bgl* I and *Xho* I, but digested by *Pst* I (233 bp + 55 bp) (*Bgl* I/*Xho* I/*Pst* I = −/−/+). A CSFV isolate correspond to

viruses with amplified products digested by *Bgl* I (two DNA fragments of 42 and 246 bp), *Xho* I (117 bp + 171 bp) and *Pst* I (233 bp + 55 bp) (*Bgl* I/*Xho* I/*Pst* I = +/+/+) (Vilcek et al., 1994; Harpin et al., 1995; Paton et al., 1995).

2.5. Nucleotide sequencing and computer analysis

The amplified products obtained after RT-PCR using primers 324/326 were purified (Prep-A-Gene DNA Purification System, Bio Rad) and directly sequenced by forward and reverse PCR primers using the fmol DNA Cycle Sequencing System (Promega) according to manufacturer's protocol. Both strands of each PCR product were sequenced in triplicate from amplified products obtained from individual amplifications. The sequences obtained have been deposited in the GenBank data library (see accession numbers in Table 1). The portion examined for 5′UTR corresponds to positions 142–371 in BVDV NADL strain. The nucleotide sequences from the 5′UTR of pestivirus strains not collected in this work were taken from databases.

Sequences were aligned using the ClustalW software (Thompson et al., 1994). A matrix of distances for Kimura's two-parameter model was then generated (Kimura, 1980) and used to compute neighbor-joining phylogenetic trees. The robustness of the phylogenetic analysis and significance of the branching order were determined by a bootstrap analysis carried out on 100 replicates. These methods were implemented with software from the MEGA program (Kumar et al., 1994).

2.6. Antigenic characterization

The isolates were characterized antigenically by cross-neutralization, using virus-specific antisera raised in rabbits. Virus-specific antisera were raised against reference strain NADL (BVDV-1a); and Chilean isolates CH511 (BVDV-1c) and CH470 (BVDV-2). Sera were heat-inactivated (56 °C for 30 min) prior to virus neutralization (VN) assays performed in microtiter plates. Then 50 μl of culture medium, containing 100 $TCID_{50}$ (50% tissue culture infectious doses) of each virus, was added to 50 μl of two-fold dilutions of antisera in medium. After 1 h at 37 °C, 100 μl of a suspension of 1.0×10^5 cells/ml was added to each well and the plates were incubated at 37 °C. Individual serum samples were initially titrated against their homologous viruses and subsequently titrated against heterologous viruses in a standard VN assay. Readings were performed after 72 h of incubation. Neutralization was monitored by microscopic examination of cytophatic effect (CPE) for the cytophatic (CP) strain (NADL) or by immunoperoxidase (IPEX-BVD; CVL, Weybridge, UK) for the non-cytopathic (NCP) viruses: CH113 (BVDV-1b), CH511 (BVDV-1c), CH470 (BVDV-2) and CH809 (BVDV-2). The VN tests were performed at the same time, using the same preparation of cells. The titer corresponding to each virus-serum combination was expressed as the reciprocal of the serum dilution capable of inhibiting viral replication in 50% of the wells.

3. Results and discussion

In this work we analyzed the genetic heterogeneity of 33 BVDV isolates from Chile in order to test for the prevalence of the different genotypes and subgroups in Chilean cattle and contribute to the understanding of the evolution and epizootiology of these viruses.

The genetic analysis was performed on the basis of enzyme digestion and differential RT-PCR amplification of the 5′UTR of the 33 isolates obtained from

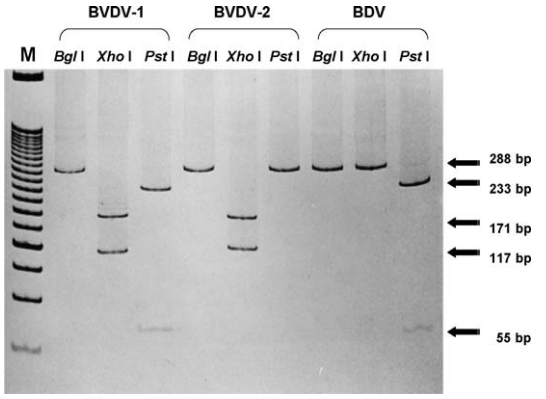

Fig. 1. Polyacrylamide gel electrophoresis showing the three representative digestion patterns of amplified products obtained by RT-PCR amplification by 324/326 primers from field isolates of pestivirus, using the restriction enzymes *Bgl* I, *Xho* I or *Pst* I. M: 25 bp ladder (Gibco-BRL). The arrowed bands show the DNA amplified product obtained by RT-PCR (288 bp) and the DNA fragments obtained by digestion with the restriction enzymes.

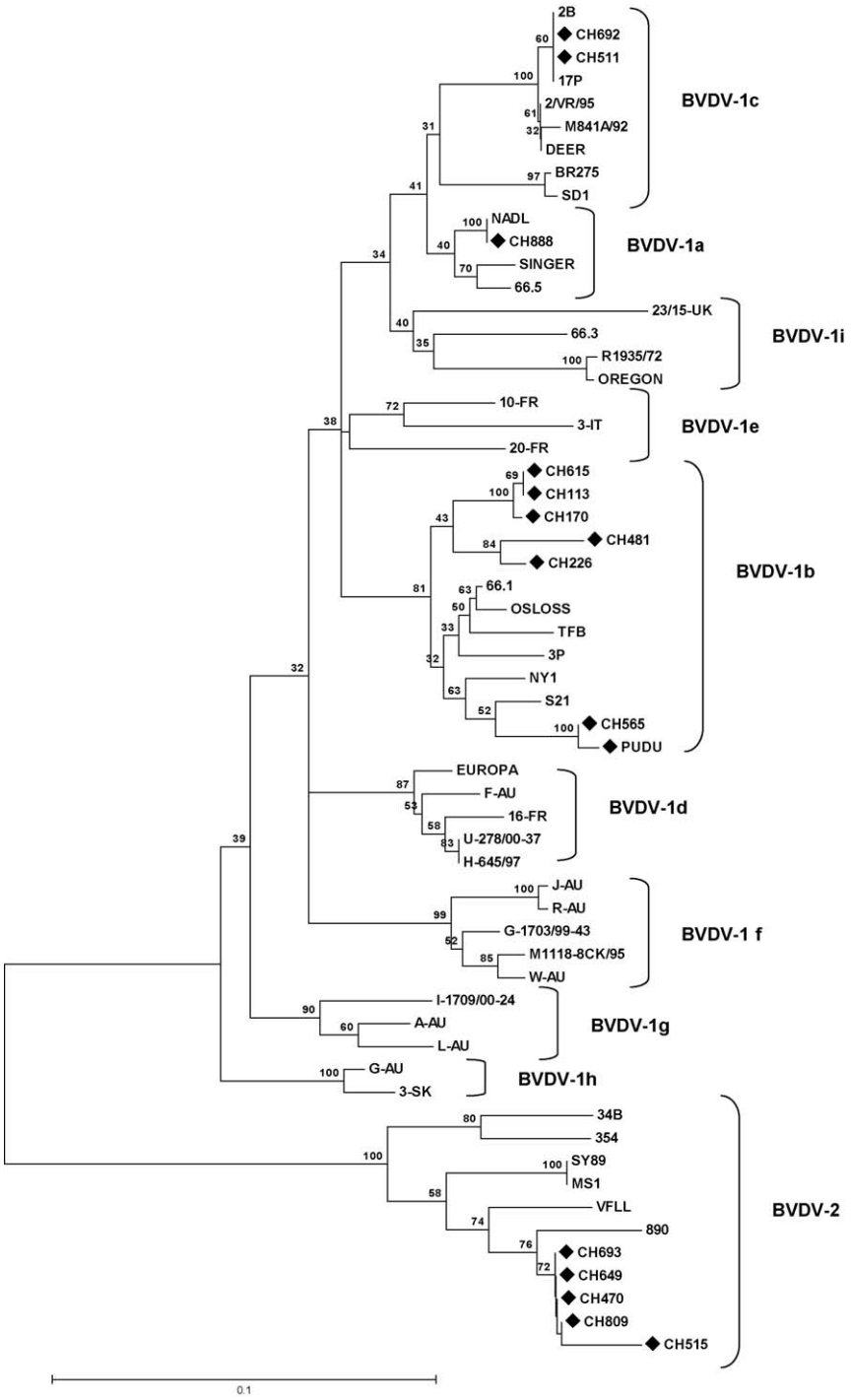

Fig. 2. Phylogenetic tree of 5′UTR sequences from Chilean BVDV isolates and other BVDV isolates representing the major BVDV genetic types. **BVDV-1a**: NADL (accession number M31182); Singer (L32875); SD-1 (M96751); BR275 (U94915); 66.5 (AF244961); Oregon (AF091605); R1935/72 (U94916); 66.3 (AF244967). **BVDV-1b**: Osloss (M96687): NY-1 (L32879); 3P (AF244968); TFB (AF244971); 66.1

animals of a diverse clinical status between 1993 and 2001 (Table 1).

The 33 pestiviruses were amplified using 324/326 primers giving the expected fragment of 288 pb in length, and then all the amplified products were digested with *Bgl* I, *Xho* I and *Pst* I restriction enzymes. According to digestion pattern observed, 14 viruses were identified as BVDV-1 (*Bgl* I/*Xho* I/*Pst* I = −/+/+); 17 viruses as BVDV-2 (*Bgl* I/*Xho* I/*Pst* I = −/+/−); and two viruses (CH511 and CH692) as BDV (*Bgl* I/*Xho* I/*Pst* I = −/−/+) (Fig. 1). None of the pestivirus isolates presented a digestion pattern characteristic of the CSFV isolates (*Bgl* I/*Xho* I/*Pst* I = +/+/+) (Vilcek et al., 1994; Harpin et al., 1995; Paton et al., 1995).

When all 33 pestiviruses were analyzed by differential RT-PCR, all the BVDV isolates identified as BVDV-1 and BVDV-2 by restriction enzymes were confirmed by differential RT-PCR. BVDV-1 viruses were amplified by primers 324/326 giving the expected 288 bp DNA fragment, and were not amplified by primers JP1/JP2. On the other hand, BVDV-2 viruses were amplified by primers 324/326 and primers JP1/JP2, giving the expected 117 DNA fragment. According to this, the viruses classified as BDV by restriction enzymes (CH511 and CH692) were typed as BVDV-1 viruses by differential RT-PCR.

Part of the 5′UTR from pestiviruses amplified with the panpestivirus-specific primers 324/326 was directly sequenced after a purification procedure. The viruses sequenced included isolates of BVDV-1 (CH113, CH170, CH226, CH481, CH565, CH615, and CH888), and BVDV-2 (CH470, CH515, CH649, CH693 and CH809) genotypes, determined previously by restriction enzymes and differential RT-PCR, and viruses CH511 and CH692. These viruses were selected considering different places of origin throughout central and south of Chile, and different clinical disorders observed in the animals infected with the viruses.

The genetic analysis of the 5′UTR region, corresponding to nucleotides 142–371 of the NADL strain, between Chilean pestiviruses, reference strains and sequences published by other groups confirmed the genetic typing of the Chilean isolates obtained by restriction enzymes and differential RT-PCR, and identified isolates CH511 and CH692 as BVDV-1 viruses. This demonstrated, for the first time, the presence of isolates belonging to BVDV-1 and BVDV-2 genotypes in Chilean cattle.

Then, the results showed that differential RT-PCR is a effective tool to perform a fast and low cost genetic typing of BVDV viruses that identify viruses as BVDV-1 or BVDV-2. This is particularly useful in regions of the world where direct phylogenetic analysis cannot be performed because of their high cost or the technical expertise required.

The range of percentages of similarity of the Chilean isolates was 88.6–100% between BVDV-1 isolates, 98.2–100% between BVDV-2 isolates and 73.3–78.2% between the BVDV-1 and BVDV-2 isolates.

A phylogenetic tree of the BVDV isolates was constructed (Fig. 2). Among the Chilean BVDV-1 isolates analyzed ($n = 9$), most of them ($n = 6$) belonged to BVDV-1b subgroup (CH113, CH170, CH226, CH481, CH565, CH615), one isolate (CH888) belonged to BVDV-1a subgroup, and two isolates (CH511 and CH692) belonged to BVDV-1c subgroup.

Interestingly, CH888 isolate has a sequence similarity of 100% against the NADL strain at the 5′UTR. A similar situation has been observed with the Brazilian strains BR275 and R1935/72. The BR275 strain has a sequence similarity of 99.5% against SD-1 strain, whereas the R1935/72 strain has a sequence similarity of 99.5% against the Oregon strain. NADL,

(AF244953); S21 (AF244963); Pudu (AY679726) (Pizarro-Lucero et al., 2005). **BVDV-1c**: Deer (AB040132); 2/Vr/95 (AJ293594); M841A/92 (U97462); 2B (AF244957); 17P (AF244954). **BVDV-1d**: 16-FR (AF298056); F-AU (AF298065); U-278/00-37 (AY323887); H-645/97 (AY323888); EUROPA (AB000898). **BVDV-1e**: 3-IT (AF298062); 10-FR (AF298054); 20-FR (AF298058). **BVDV-1f**: J-AU (AF298067); R-AU (AF298071); W-AU (AF298073); G-1703/99-43 (AY323876); M1118-8CK/95 (U97421). **BVDV-1g**: A-AU (AF298064); L-AU (AF298069); I-1709/00-24 (AY323877). **BVDV-1h**: G-AU (AF298066); 3-SK (AF298068). **BVDV-1i**: 23/15-UK (AF298059). **BVDV-2**: 890 (U18059); MS-1 (AB019688); SY-89 (AB019689); VFLL (AB019687); 34B (AF244952); 354 (AF244959). The aligned sequences correspond to nucleotides 142–371 in the NADL sequence. The viral genotypes and subgroups are indicated. Phylogenetic analysis was done by the neighbour-joining method using MEGA (Kumar et al., 1994). The robustness of the phylogenetic analysis and significance of the branching order were determined by bootstrap analysis carried out on 100 replicates. Evolutionary distances between sequences were estimated by the Kimura 2-parameter method (Kimura, 1980). The Chilean isolates are rhombus marked.

Oregon and SD-1 strains are used for the formulation of live and inactivated vaccines against BVDV, therefore, CH888, BR275 and R1935/72 could be post-vaccinal isolation of these lab strains. Nevertheless, the precise origin of the strain CH888 remains to be established.

The analysis of the nucleotide sequence of the 5′UTR of the isolates CH511 and CH692, showed that the two isolates have replaced a G with a T in position 229, according to NADL genome sequence, and had lost the restriction site for the *Xho* I enzyme. Interestingly, it was observed that all the BVDV-1c viruses analyzed have suffered the same change. This characteristic could be useful in order to identify the viruses that belong to BVDV-1c subgroup.

The Chilean pestiviruses CH511 and CH692 identified as BVDV-1c viruses showed 100% similarity to the Argentinean isolates (2B, 17P) and 99% similarity to Italian and Mozambique bovine and deer isolates. Given the differences in geographical location (Chile, Argentina, Italia, Mozambique) and specie origin (deer or bovine), the extremely high

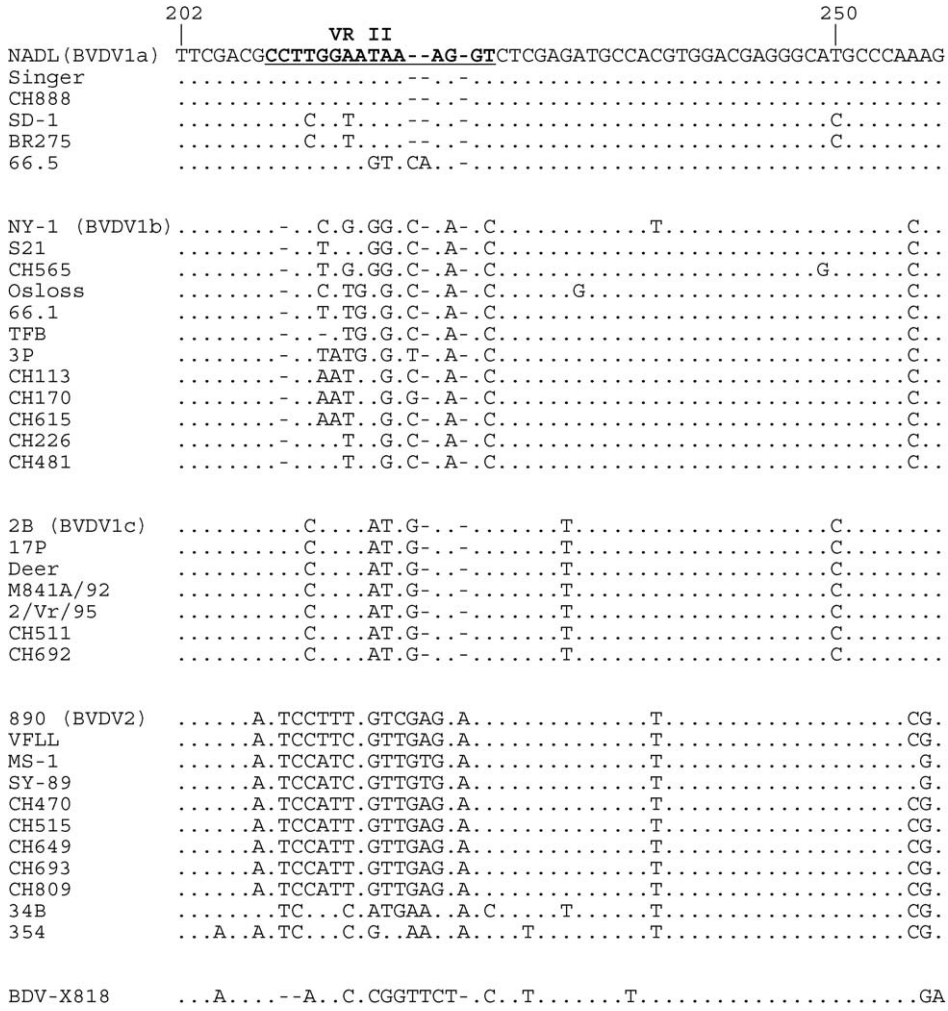

Fig. 3. Alignment of 5′UTR nucleotide sequences from Chilean BVDV isolates and other BVDV isolates of the same genotypes and subgroups. The aligned sequences correspond to nucleotides 202–258 in the NADL sequence. Dots represent nucleotides that are identical to NADL, uppercase letters represent a nucleotide substitution and the dash indicates a putative nucleotide deletion. Variable region II (nucleotides 209–223 of BVDV NADL sequence) is indicated (Deng and Brock, 1993).

genomic similarities that exist among all the viruses in this subgroup is remarkable. All of this suggests that the high genomic stability observed in these viruses would be a specific and important characteristic of this genetic group of viruses (BVDV-1c).

Interestingly, phylogenetic analysis placed isolates Oregon (USA), 66.3 (Argentina), and R1935/72 (Brasil), previously classified as BVDV-1a viruses, in BVDV-1i subgroup, together with the virus 23/15-UK, a UK isolate (Vilcek et al., 2001). This demonstrated that group BVDV-1i of viruses described up until now only in UK, should be circulating in different places of the world.

In order to study the genetic characteristics of the Chilean isolates at the nucleotide level, the sequences were aligned and the nucleotidic substitution patterns in the variable region II (NSPVR-II), a region particularly rich in informative bases (Deng and Brock, 1993), were shown in relation to the NADL genome sequence (Fig. 3).

Surprisingly, a unique NSPVR-II in all the viruses that belong to subgroup BVDV-1c could be observed, and was clearly different from the NSPVR-II showed by BVDV-1a viruses. This support the surprisingly genomic stability of BVDV-1c viruses and their classification in a different subgroup as suggested by Luzzago et al. (2001), Jones et al. (2001), and Baule et al. (1997).

It is concluded that from 33 isolates obtained from healthy and clinically affected animals, 16 animals were infected with BVDV-1 isolates (48.5%), and 17 were infected with BVDV-2 isolates (51.5%). These percentages are very similar to those observed in the USA, where BVDV-2 isolates range from 40–54% according to different reports (Ridpath et al., 1994, 2000; Bolin and Ridpath, 1998; Ridpath and Bolin, 1998), and different from reports from Europe, Brazil, Argentina, South Africa and Japan that show BVDV-2 has either not been detected or the prevalence is very low with respect to the BVDV-1 genotype (Baule et al., 1997; Wolfmeyer et al., 1997; Nagai et al., 1998; Shimazaki et al., 1998; Vilcek et al., 1999, 2003; Flores et al., 2000; Jones et al., 2001; Couvreur et al., 2002). This probably means that viruses belonging to BVDV-2 genotype have been circulating for a long time in bovines in Chile.

Antisera raised against NADL (BVDV-1a), CH511 (BVDV-1c) and CH470 (BVDV-2) strains were used for cross-neutralization studies with NADL, CH113 (BVDV-1b), CH511, CH470 and CH809 (BVDV-2) strains, representing each genotype and subgroup (Table 2). NADL antisera showed a high serological cross-reactivity with the CH113 strain (difference between titers, 1.4-fold), little serological cross-reactivity with the CH470 and CH809 strains (difference between titers, 22.5- and 61.0-fold, respectively), and non-detectable serological cross-reactivity with the CH511 strain. On the other hand, CH511 antisera showed little serological cross-reactivity with NADL, CH113, CH470 and CH809 strains (difference between titers, 11.3-, 45-, 11.3- and 22.5-fold, respectively). The CH470 antisera showed little serological cross-reactivity with NADL and CH113 strains (difference between titers, 22.9 and 11.6, respectively), and non-detectable serological cross-reactivity with CH511 and CH809 strains. These results allowed us to separate the BVDV strains into four serologically distinct groups of viruses: group 1: NADL and CH113 strains, group 2: CH511 strain, group 3: CH470 strain, and group 4: CH809 strain.

Table 2
Comparison of BVDV isolates based on Virus-Neutralizing (VN) titers using polyclonal antisera[a]

Virus	Anti-NADL	Antiserum anti-CH511	Anti-CH470
NADL (BVDV-1a)	**360**	16	5.6
CH113 (BVDV-1b)	256	4	11
CH511 (BVDV-1c)	<2	**180**	<2
CH470 (BVDV-2)	16	16	**128**
CH809 (BVDV-2)	5.9	8	<2

[a] VN titers are expressed as the reciprocal of the highest dilution capable of neutralizing viral replication in 50% of the wells. Virus replication was ascertained by monitoring the cytopathic effect (NADL) or by IPI (CH113, CH511, CH470, and CH809).

The high serological cross-reactivity founded between BVDV-1a and BVDV-1b viruses and the low serological cross-reactivity founded between the BVDV-1a and BVDV-1b viruses with the BVDV-2 viruses are in agreement with previously reported results from other authors, which could explain the failure of vaccines to protect against all the field isolates (Howard et al., 1987; Bolin et al., 1988, 1991; Pellerin et al., 1994; Wolfmeyer et al., 1997; Ridpath et al., 2000; Couvreur et al., 2002). Nevertheless, the non-detectable or very low serological cross-reactivity that exists between isolate CH511 (BVDV-1c) and the

viruses that belong to BVDV-1a, BVDV-1b and BVDV-2 subgroups is surprising, and the same occurred between the Chilean BVDV-2 isolates.

These results revealed marked antigenic differences between the Chilean isolates, which are probably at the genomic level of E2 since this glycoprotein is the major viral protein mediating neutralization of BVDV (Meyers and Thiel, 1996). These findings have important implications for vaccine development and immunization strategies. The extremely low capacity of antisera against NADL, strain used extensively in the vaccine formulation, to neutralize BVDV-2 and BVDV-1c isolates, predicts the low efficiency of classical BVDV vaccines toward these strains and may indicate the need to formulate vaccines that include these isolates.

Both genotypes were found distributed in different places throughout Central Chile (data not shown). The widespread prevalence of virus variants may be explained by the intensive movements of cattle between farms within the same or different regions and by poor BVDV control procedures.

A relationship between clinical syndrome and genotype could not be established. Viruses belonging to both genotypes are distributed among healthy and sick cattle, thus supporting the idea postulated by some authors that avirulent BVDV-2 do exist and may predominate over virulent BVDV-2 in nature (Wolfmeyer et al., 1997; Flores et al., 2000; Ridpath et al., 2000). Nevertheless, the CH809 strain of BVDV-2 genotype was isolated from an animal that showed clinical signs highly suggestive of mucosal disease, but without the CP counterpart, thereby suggesting that BVDV-2 hypervirulent strains are circulating in the chilean cattle.

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