Genoepidemiological Evaluation of *Bovine viral diarrhea virus 2* Species Based on Secondary Structures in the 5' Untranslated Region

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ABSTRACT. Bovine viral diarrhea virus 2 (BVDV-2) strains demonstrated in cattle, sheep, and adventitious contaminants of biological products have been evaluated by the palindromic nucleotide substitutions (PNS) method at the three variable loci (V1, V2 and V3) in the 5' untranslated region (UTR), to determine their taxonomical status. Variation in conserved genomic sequences was used as parameter for epidemiological evaluation of the species in relation with geographical distribution, animal host and virulence. Four genotypes, BVDV-2a, BVDV-2b, BVDV-2c, and BVDV-2d have been identified within the species. Taxonomical segregation corresponded to geographical distribution of genotype variants. Genotype 2a was present worldwide, and was the only circulating also in sheep, in addition to cattle. Genotypes 2b, 2c and 2d were restricted to South America. Contamination of biological products was related to genotypes 2a and 2d. Genetic variation could be related with chronological diffusion of the BVDV-2 species variants in different geographic areas. Chronologically, the species emerged in North America in 1978, spreading in UK and Japan, continental Europe, South America and New Zealand. Correlation between clinical features related with isolation of BVDV-2 strains and genetic variation indicated that sub-genotype 1, variant 4 of genotype 2a was related with hemorrhagic syndrome. These observations suggest that evaluation of genomic secondary structure, by identifying markers for expression of virus biological activities and species evolutionary history, may be applied as useful tool for epidemiological evaluation of the BVDV-2 species, and possibly for other species of the genus *Pestivirus*.

KEY WORDS: BVDV-2, genoepidemiology, palindromic nucleotide substitutions, pestivirus.

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Bovine viral diarrhea virus 2 (BVDV-2), represents an established species of the genus *Pestivirus* of the family *Flaviviridae*, with *Bovine viral diarrhea virus 1* (BVDV-1), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) and a tentative “Giraffe” species [12]. BVDV-2 species includes the strains isolated from outbreaks hemorrhagic syndrome characterised by thrombocytopenia and high mortality in U.S.A. and Canada [5, 25, 27]. Hyper-virulent strains 890 and CD-87 have been previously included in BVDV species in a separate cluster [25, 28]. The hemorrhagic syndrome has been reported also in European cattle [7, 24, 36]. Different strains were also isolated in bovine animals showing mild clinical forms [22, 35]. Furthermore, the presence of the species was observed in biological products as adventitious contaminant [4, 16, 18, 20], indicating the risk of iatrogenic infection, demonstrated by the recent occurrence of a serious accident in the Netherlands among cattle, who received a live virus vaccine contaminated with pestivirus [11].

The *Pestivirus* genome, single-stranded, positive polarity RNA, is composed by a sequence of about 12,500 nucleotides, which can be divided into three regions: a 5'-untranslated region (UTR) containing an internal ribosomal entry site (IRES), a single large open reading frame encoding a polyprotein, and a 3'-UTR. The 5'-UTR is highly con-

served among all members within the genus *Pestivirus*, thus being useful for the characterization of species or genotypes. The primary structure analysis, by sequence alignment and construction of phylogenetic trees, is the most common method for the classification of the *Pestivirus* isolates. However, it is relatively easy to predict the secondary structure, according to the most probable nucleotide binding, with lowest folding energies. The secondary structure of the 5'-UTR can be divided into four domains, A-D, with domain D encompassing the two thirds in the 3' region of the 5'-UTR predicted to fold into a complex palindromic stem-loop structure [9, 16], a critical region of the 5'-UTR, which is responsible for translational, transcriptional and replicational events in pestiviruses. Therefore, random mutations at the 5'-UTR have a high probability of incompatibility with viral survival. Thus stable nucleotide variations at this level assume high importance in terms of virus evolutionary history. The nucleotide substitutions of the IRES in the 5'-UTR were supposed to be responsible for the virulence of poliovirus [26]. Mutations or nucleotide substitutions at palindromic IRES regions will affect efficiency translation from RNA to protein. Thus low translation might reduce virulence, and high translation increase virulence. Due to genetic structure similarity with pestiviruses, this concept was proposed for BVDV-2 by Topliff and Kelling [35]. The authors reported a relatedness between high virulence and two specific nucleotides, uracil and cytosine located at position 219 and 278 of the genomic sequence (based on BVDV-1 strain Osloss) and low viru-

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lence with cytosine and uracil at position 219 and 278, respectively. Nucleotide sequences at the three variable loci, V1, V2 and V3, in the 5'-UTR of pestiviruses have been shown to be palindromic and capable of forming a stable stem-loop structure peculiar to each *Pestivirus* species. Nucleotide substitutions in the stem regions always occur to maintain the palindromic sequence and thereby form a stable stem-loop structure. Thus, this type of mutation was referred to palindromic nucleotide substitutions (PNS). Based on the above mentioned considerations, the observation of nucleotide variations among virus strains at the level of the three specific palindromes in the 5'-UTR has been conceived as method for genotyping [17]. The method named PNS analysis appeared to be simple and practical, showing comparable results with other procedures based on the primary structure comparison. The results of the PNS method can be essentially qualitative and provide the exact species classification of an isolate. Thus, this method may help to clarify species and genotype boundaries, due to the exclusive consideration of strategic and highly conserved regions, and consequently helps to avoid unclear classification.

In the present study, BVDV-2 *Pestivirus* strains, isolates from cattle and sheep, and adventitious contaminants of biological products, have been evaluated with PNS analysis in the 5'-UTR to determine relation between genotypic variations and epidemiological observations.

MATERIALS AND METHODS

The nucleotide sequences in the 5'-UTR of seventy-three BVDV-2 species strains were obtained from the DNA databases or provided directly from the authors via personal communication (sequences not deposited by Beer, Vilcek and Ridpath; sequences defined as ALIGN_000012 by Couvreur and available on the EMBL web site) (Table 1). According to the geographic origin of the virus isolates fifteen were reported from North America, and twelve from South America, thirty two from Europe, thirteen from Japan, and one from New Zealand. Eight strains originated from sheep, isolated from outbreaks of border disease. Twelve strains were detected as contaminants from cell cultures and biologicals. The strain IT-1732 (AJ416018) (Muscillo, unpublished) was isolated from a bayovac IBR-

Table 1. BVDV-2 strains evaluated according to secondary structure nucleotide changes. ND: not determined

Genotype	Strain	Origin	Country	Year of isolation	Clinical presentation	Accession	Reference
BVDV-2a1.1	5521-95	Cattle	USA	1995	Fetal abortion	AF039174	Topliff & Kelling, 98
BVDV-2a1.1	713-2	Cattle	USA	1982	Fetal abortion	AF039177	Topliff & Kelling, 98
BVDV-2a1.1	97/730	Cattle	New Zealand	1997	Data not available	AF026770	Vilcek <i>et al.</i> , unpublished
BVDV-2a1.1	BSE1239	Cattle	Belgium	1996	Neurological symptoms	ALIGN_000012	Couvreur <i>et al.</i> , 02
BVDV-2a1.1	UVR420	Cattle	Belgium	1991	Calf intestine	ALIGN_000012	Couvreur <i>et al.</i> , 02
BVDV-2a1.2	BD-78	Sheep	USA	1978	Border disease	U18330	Sullivan <i>et al.</i> , 94
BVDV-2a1.2	C413	Sheep	USA	2002	Border disease	AF002227	Chen & Berry, unpublished
BVDV-2a1.3	167 237	Sheep	UK	1987	Border disease	U65055	Vilcek <i>et al.</i> , 97
BVDV-2a1.3	168 149	Sheep	UK	1987	Border disease	U65056	Vilcek <i>et al.</i> , 97
BVDV-2a1.3	173 157	Sheep	UK	1987	Border disease	U65058	Vilcek <i>et al.</i> , 97
BVDV-2a1.3	175 375	Sheep	UK	1987	Border disease	U65059	Vilcek <i>et al.</i> , 97
BVDV-2a1.3	BSE921	Cattle	Belgium	1995	Neurological symptoms	ALIGN_000012	Couvreur <i>et al.</i> , 02
BVDV-2a1.3	Lees	Sheep	UK	1985	Border disease	U65051	Vilcek <i>et al.</i> , 97
BVDV-2a1.3	CPA	Contaminant	Japan	1993	Not applicable	D50812	Harasawa & Mizusawa, 95
BVDV-2a1.3	CPAE	Contaminant	Japan	1993	Not applicable	D50813	Harasawa & Mizusawa, 95
BVDV-2a1.3	EBTr	Contaminant	Japan	1993	Not applicable	D50817	Harasawa <i>et al.</i> , 93
BVDV-2a1.3	HE727	Contaminant	Japan	1995	Not applicable	D31807	Harasawa & Mizusawa, 95
BVDV-2a1.3	MMR-T	Contaminant	Japan	1993	Not applicable	D26052	Harasawa & Tomiyama, 94
BVDV-2a1.3	MP	Contaminant	Belgium	2002	Not applicable	ALIGN_000012	Couvreur <i>et al.</i> , 02
BVDV-2a1.3	Parvo	Contaminant	Japan	1994	Not applicable	D26614	Harasawa, 94
BVDV-2a1.3	Rubella	Contaminant	Japan	1993	Not applicable	D26048	Harasawa & Tomiyama, 94
BVDV-2a1.4	11/Mi/97	Cattle	Italy	1997	Data not available	AJ293603	Luzzago <i>et al.</i> , 00
BVDV-2a1.4	15-103	Cattle	France	1994	Data not available	AF298055	Vilcek <i>et al.</i> , 01
BVDV-2a1.4	17583-97	Cattle	USA	1997	Acute BVD	AF039176	Topliff & Kelling, 98
BVDV-2a1.4	23025	Cattle	USA	1993	Hemorrhagic syndrome	AF039172	Topliff & Kelling, 98
BVDV-2a1.4	37Gr	Cattle	Austria	98-00	Acute diarrhea	not deposited	Vilcek <i>et al.</i> , 03
BVDV-2a1.4	7937	Cattle	USA	1988	Persistent infection	AF039175	Topliff & Kelling, 98
BVDV-2a1.4	AZ Spl	Cattle	USA	60-93	Hemorrhagic syndrome	not deposited	Ridpath <i>et al.</i> , 94
BVDV-2a1.4	BSE341	Cattle	Belgium	1991	Neurological symptoms	ALIGN_000012	Couvreur <i>et al.</i> , 02
BVDV-2a1.4	IT-1732	Contaminant	Italy	1999	Acute BVD	AJ416018	Muscillo, unpublished
BVDV-2a1.4	Kosice	Cattle	Slovakia	2002	Fatal course	not deposited	Vilcek <i>et al.</i> , 02
					Malformations, hemorrhagies		
BVDV-2a1.4	MAD Spl	Cattle	USA	60-93	Hemorrhagic syndrome	not deposited	Ridpath <i>et al.</i> , 94
BVDV-2a1.4	MN Fetus	Cattle	USA	60-93	Hemorrhagic syndrome	not deposited	Ridpath <i>et al.</i> , 94

Marker vaccine (Bayer), responsible for outbreaks in the Netherlands and Italy [11]. The strain WG4622 [ALIGN_000012] [8] was isolated from the same batch of vaccine. Strains MMR-T [D26052], Rubella [D26048] [16, 20] and HE727 [D31807] [19], were isolated from Japanese commercial virus vaccines and interferons for human use. Strain 354 [AF244959] [21] was isolated in Argentina.

The secondary structures were predicted according to the algorithm of Zuker and Stiegler [40]. The minimum free energy was calculated by the method of Freier *et al.* [14]. Three variable regions, V1, V2 and V3, at the 5'-UTR were used for genotyping based on palindromic nucleotide substitutions [17]. In each variable locus, nucleotide base pairs have been defined as positions starting their numbering from the bottom of the stems of the palindromes. Sequences

were aligned according to secondary structure to evaluate nucleotide changes in relation with geographic distribution, animal hosts and virulence, identifying specific PNS markers.

RESULTS

The evaluation of the BVDV-2 *Pestivirus* strain 5'-UTR nucleotide sequences, according to PNS analysis allowed the identification of specific nucleotide changes among genotype variants of the species, related with geographic segregation, as well as animal host origin and virulence.

Characteristic base pairings common to BVDV-2 species were observed in all tested isolates. The application of the PNS method showed four genotypes, BVDV-2a, BVDV-2b,

Table 1. (cont'd)

Species	Strain	Origin	Country	Year of isolation	Clinical presentation	Accession	Reference
BVDV-2a1.4	NY93	Cattle	USA	1993	Acute BDV	AF039173	Topliff & Kelling, 98
BVDV-2a1.4	Q126	Cattle	Canada	1994	Hemorrhagic syndrome	L32890	Pellerin <i>et al.</i> , 94
BVDV-2a1.4	V-FLL	Contaminant	Japan	1999	Not applicable	AB019687	Sakoda <i>et al.</i> , 99
BVDV-2a1.4	WG4622	Contaminant	Netherland	1999	Letal acute BVD	ALIGN_000012	Couvreur <i>et al.</i> , 02
BVDV-2a1.4	WVD829	Cattle	Belgium	1994	Respiratory symptoms	ALIGN_000012	Couvreur <i>et al.</i> , 02
BVDV-2a1.5	890	Cattle	Canada	1990	Hemorrhagic syndrome	L32886	Pellerin <i>et al.</i> , 94
BVDV-2a1.6	104/98	Cattle	Germany	1998	Mild symptoms	AJ304381	Tajima <i>et al.</i> , 01
BVDV-2a1.6	B52-2	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a1.6	CD87	Cattle	Canada	1987	Hemorrhagic syndrome	L32887	Pellerin <i>et al.</i> , 94
BVDV-2a1.6	i4083	Cattle	Argentina	2001	Mild symptoms	AF417995	Jones <i>et al.</i> , unpublished
BVDV-2a1.6	i61380	Cattle	Argentina	2001	Mild symptoms	AF417986	Jones <i>et al.</i> , unpublished
BVDV-2a1.6	i628	Cattle	Argentina	2001	Mild symptoms	AF417985	Jones <i>et al.</i> , unpublished
BVDV-2a1.6	Munich 1	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a1.6	Munich 2	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a1.6	4-5174	Cattle	France	1994	Data not available	AF298063	Vilček <i>et al.</i> , 01
BVDV-2a2	B45-5	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a2	B50-5	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a2	B5-4	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a2	B77-5	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a2	Giessen-1	Cattle	Germany	1996	Fatal mucosal disease	AF104030	Becher <i>et al.</i> , 99
BVDV-2a2	Munich 3	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a2	17011-96	Cattle	USA	1996	Fetal abortion	AF039179	Topliff & Kelling, 98
BVDV-2a2	AF112	Cattle	Germany	92-99	Mild symptoms	not deposited	Beer <i>et al.</i> , 02
BVDV-2a2	BS-95-II	Cattle	Italy	1995	Data not available	AJ288903	Luzzago <i>et al.</i> , 00
BVDV-2a2	MS-1	Cattle	Japan	1999	Persistent infection	AB019688	Sakoda <i>et al.</i> , 99
BVDV-2a2	SW90	Cattle	Japan	1990	Mild symptoms	AB003622	Nagai <i>et al.</i> , 01
BVDV-2a2	SY-89	Cattle	Japan	1989	Persistent infection	AB019689	Sakoda <i>et al.</i> , 99
BVDV-2a2	OY89	Cattle	Japan	1989	Persistent infection	AB003621	Nagai <i>et al.</i> , 98
BVDV-2a2	TC Shinozaki	Cattle	Japan	1992	Respiratory symptoms	AB04267	Nagai <i>et al.</i> , 01
BVDV-2b1.1	VS-63	Cattle	Brazil	1996	Healthy fetus	AF410789	Flores <i>et al.</i> , 02
BVDV-2b1.2	34b	Cattle	Argentina	1991	Fetus	AF244952	Jones <i>et al.</i> , 01
BVDV-2b1.2	ncp7	Cattle	Argentina	1993	Fetus	AY443026	Jones <i>et al.</i> , 01
BVDV-2b1.3	VS-123.4	Cattle	Brazil	1996	Healthy fetus	AF410790	Flores <i>et al.</i> , 02
BVDV-2b1.4	LV-96	Cattle	Brazil	1996	Chronic gastroenteric	AF410787	Flores <i>et al.</i> , 02
BVDV-2b1.4	VS-260	Cattle	Brazil	1997	Gastroenteric symptoms	AF410788	Flores <i>et al.</i> , 02
BVDV-2b2	Soldan	Cattle	Brazil	1991	Mucosal disease	U94914	Canal <i>et al.</i> , unpublished
BVDV-2c	i33283	Cattle	Argentina	2001	Mild symptoms	AF417996	Jones <i>et al.</i> , unpublished
BVDV-2d	354	Contaminant	Argentina	1995	Not applicable	AF244959	Jones <i>et al.</i> , 01
BVDV-2 ND	59386	Sheep	UK	1994	Border disease	U17146	Becher <i>et al.</i> , 94
BVDV-2 ND	SCP	Contaminant	UK	1994	Not applicable	U17148	Becher <i>et al.</i> , 94

Table 2. Geographical distribution and origin of isolation of BVDV-2 genotypes, subgenotypes and variants * contaminants only; Yes: detected; No: not detected

Genotypes	Subgenotype	Variant	North America	South America	Europe	Asia	Austral-asia
BVDV-2a	1	1	USA		Belgium		New Zealand
BVDV-2a	1	2	USA				
BVDV-2a	1	3			Belgium, UK	Japan*	
BVDV-2a	1	4	Canada, USA		Austria, Belgium, France, Italy, Netherlands*, Slovakia	Japan	
BVDV-2a	1	5	Canada				
BVDV-2a	1	6	Canada	Argentina	France, Germany		
BVDV-2a	2	—	USA		Germany, Italy	Japan	
BVDV-2b	1	1		Brazil			
BVDV-2b	1	2		Argentina			
BVDV-2b	1	3		Brazil			
BVDV-2b	2	—		Brazil			
BVDV-2c	—	—		Argentina			
BVDV-2d	—	—		Argentina*			
Genotypes	Subgenotype	Variant	Cattle	Sheep	Contaminant		
BVDV-2a	1	1	Yes	No	No		
BVDV-2a	1	2	No	Yes	No		
BVDV-2a	1	3	Yes	Yes	Yes		
BVDV-2a	1	4	Yes	No	Yes		
BVDV-2a	1	5	Yes	No	No		
BVDV-2a	1	6	Yes	No	No		
BVDV-2a	2	—	Yes	No	No		
BVDV-2b	1	1	Yes	No	No		
BVDV-2b	1	2	Yes	No	No		
BVDV-2b	1	3	Yes	No	No		
BVDV-2b	2	—	Yes	No	No		
BVDV-2c	—	—	Yes	No	No		
BVDV-2d	—	—	No	No	Yes		

BVDV-2c, and BVDV-2d. Two strains from Argentina were classified as genotypes BVDV-2c and BVDV-2d, showing unexpected base pair changes, suggesting their intermediate phylogenetic position between North American, European and Asian strains and South American ones. Genotype 2b, 2c and 2d South American strains were characterized by GA or GG or AC in position 17 of V1 locus (V1/17), V1/18 GG or GA and a higher V3 loop. Genotype 2c shared with 2b characteristic pairing GC in V1/12, thus in V1/16 showed CG, common pairing in strains from other continents, instead of UA pairing common to other South American strains. In addition a specific CG pairing in position 14 of V1 characterised the genotype 2c. Genotype 2d shared with 2b UA pairing in V1/16, but was divergent in V1/12 showing AU common to strains from North America, Europe or Japan. In addition, AU pairing in position 9 of the V1 locus was specific to the genotype 2d. Genotype 2a was present worldwide, and was the only circulating also in sheep, in addition to cattle, prevalent host species. Genotypes 2b, 2c and 2d were restricted to South America (Table 2).

Taxonomical segregation corresponded to geographical

distribution of genotype variants. Chronologically, the species was first detected in North America in 1978, and further in UK and Japan, thus in continental Europe, South America and New Zealand. Genetic variation could be correlated to chronological detection of the BVDV2 species variants in different geographic areas (Fig. 1). Genotype 2a subgenotype 1 variant 1, 4 and 6, first reported in North America in 1982, 1988 and 1987, appeared to circulate worldwide. Variant 1 was reisolated in continental Europe in 1991, thus in New Zealand in 1997. Variant 4, mainly highly virulent in cattle, was reported in Europe in 1991 showing mild clinical symptoms, and in 1998–00 related with high virulence. The variant was further reported in Japan as contaminant in 1999. Variant 6, related with hemorrhagic syndrome in Canada, was reisolated in Germany in 1994, and in Argentina in 2001, associated with mild symptoms. Interestingly, subgenotype 2 was first reported in Japanese cattle, and further reisolated in 1995 in Europe and in 1996 in U.S.A. Despite sequence similarities and identities, generated by animal trade, specific nucleotide changes, especially at the level of V1 and V3 loops, allowed the distinguishing of strains originated from North and South America, continen-

North America

Position	V1	1	2	3	7	8	9	12	13	14	15	16	17	18	19	20	21	22	V2	1	2	3	4	5	6	7	8	9	V3	1	2	3	4	5	6	7	8	9	10
BVDV2	AU	UA	GC	UA	CG	GC	AU	CG	UA	CG	CG					AU				CG	CG			AU	UA	GC	CG	GU	AU	GU	CG		CG	CG	AU	UG	UC	-	
Strains reported only in North America																																							
BVDV2	890												UA	UG	UC	G	-	AU			CG		CG					AC			GC							-	
BVDV2	AZ Spl		GU										AA	UG	CU	AC	G	-	AU		UG								GC			GU						-	
BVDV2	BD-78												AA	UG	UC	G	-	AU		CG		CG		CA							GC		UG					-	
BVDV2	C413												AA	UG	UC	G	-	AU		CG		CG		CA							GC		UG					-	
BVDV2	713-2												AA	UG	CC	G	-	AU				UA				UG					GC					UU		-	
Similar with 2a 1 1 New Zealand divergent V1/20 AU																																							
BVDV2	5521-95												AA	CG	CC	A	-	AU			UG										AU					UU		-	
Similar with Argentine and European 2a 1 6 V1/21A																																							
BVDV2	CD87												AA	UG	CU	A	-	GU			UA	GU								GC		GU						-	
Similar with Japanese and German 2a 2 V3/7 AC but divergent in V3/8																																							
BVDV2	17011-96												AA	UG	CU	G	-	AC			UA										AU			AC	UA	UU		-	
2a 1 4 identical with European																																							
BVDV2	MAD Spl		GU										AA	UG	UU	G	-	AU			CG										GC		UG					-	
BVDV2	MN Fetus		GU										AA	UG	UC	G	-	AU			CG										GC		UG					-	
BVDV2	NY93												AA	UG	UC	G	-	AU			CG										GC		UG					-	
BVDV2	Q126												AA	UG	UC	G	-	AU			CG										GC		UG					-	
BVDV2	23025												AA	UG	UC	G	-	AU			CG										GC		UG					-	
BVDV2	17583-97												AA	UG	UU	G	-	AU			CG										GC		UG					-	
BVDV2	7937												UA	UG	CU	G	-	AU			UG										GC							-	

South America

Position		V1	1	2	3	7	8	9	12	13	14	15	16	17	18	19	20	21	22	V2	1	2	3	4	5	6	7	8	9	V3	1	2	3	4	5	6	7	8	9	10	
BVDV2		AU	UA	GC	UA	CG	GC	AU	CG	UA	CG	CG	AU	CG	CG	AU	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG	AU	GU	CG	CG	CG	CG	CG	CG	CG	CG	CG	CG
Brazilian cattle strains characterized by V1/12 GC V1/16 UA V1/17 GA or GG or AC V1/18 GG or GA V3 higher loop similar with Argentinean divergent V2/1 AU																																									
BVDV2	LV96		GA					GC						UA	GA	GG	CG	A	-	AU		UA	UG							AU			AU						CU	U	
BVDV2	Soldan							GC						UA	GG	GA	CA	AC	A	-	AU		UA	UG								AU		UA	UA				CU	U	
BVDV2	VS-123.4							GC						UA	GA	GA	CG	GU	G	-	AU		UA	UG								AU			AU				CU	U	
BVDV2	VS-260		GU					GC						UA	GA	GG	CG	A	-	AU		UA	UG								AU			AU				CU	U		
BVDV2	VS-63							GC						UA	AC	GG	CG	A	-	AU		UA	UG									AU			AU			UU	U		
Argentinean cattle strains similar with Brazilian divergent V2/1 GU																																									
BVDV2	34b							GC						UA	GA	GA	CG	A	-	GU		UA	UG									AU					CG	CU	U		
BVDV2	nep7							GC						UA	GA	GA	CG	A	-	GU		UA	UG									AU					CG	CU	U		
Argentinean strains characterized by V1/20 AA V3 higher loop																																									
BVDV2	354							AU						UA	GA	GA	CA	AA	G	-	AC		UA	CG							AU		AU				UA		U		
BVDV2	i33283							GU		CG				GA	GA	CA	AA	GG	U		GU		UA	UG								AU		AU					CU	C	
Argentinean cattle strains similar with USA 2a 1 6 divergent V1/21, similar with European divergent V1/1 showing GU instead of AU																																									
BVDV2	i4083		GU											AA	CG	CU	G	-	GU		UA	GU									GC		GU						-		
BVDV2	i61380		GU											AA	CG	CU	G	-	GU		UA	GU									GC		GU						-		
BVDV2	i628		GU											AA	CG	CU	G	-	GU		UA	GU									GC		GU						-		

Japan

Position	V1	1	2	3	7	8	9	12	13	14	15	16	17	18	19	20	21	22	V2	1	2	3	4	5	6	7	8	9	V3	1	2	3	4	5	6	7	8	9	10
BVDV2	AU	UA	GC	UA	CG	GC	AU	CG	UA	CG	CG	AU	CG	CG	AU	CG	CG	CG	CG	CG	CG	AU	UA	GC	CG	GU	AU	GU	CG	CG	CG	AU	UG	UC	-	-	-	-	
Cattle strains characterized by V3/7 AC V3/8 UG or UU, similar with USA 17011-98 divergent in V3/8																																							
BVDV2	MS-1	.	.	.	.	.	.	.	.	.	.	.	AU	UG	CU	G	-	AC	UA	.	.	.	.	.	.	.	.	.	AU	.	.	.	AC	UU	.	-	-	-	
BVDV2	OY89	.	.	.	.	.	.	.	.	.	.	.	AU	UG	CU	G	-	AC	UA	.	.	.	.	.	.	.	.	.	AU	.	.	.	AC	.	-	-	-	-	
BVDV2	SW90	.	.	.	.	.	.	.	.	.	.	.	AU	UG	CU	G	-	AC	UA	.	.	.	.	.	.	.	.	.	AU	.	.	.	AC	.	-	-	-	-	
BVDV2	SY-89	.	.	.	.	.	.	.	.	.	.	.	AU	UG	CU	G	-	AC	UA	.	.	.	.	.	.	.	.	.	AU	.	.	.	AC	.	-	-	-	-	
BVDV2	TC Shinozaki	.	.	.	.	.	.	.	.	.	.	.	AU	UG	CU	G	-	AC	UA	.	.	.	.	.	.	.	.	.	AU	.	.	.	AC	.	-	-	-	-	
Contaminants																																							
BVDV2	CPA	.	.	.	.	.	.	.	.	.	.	.	AA	UA	CC	U	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	.	-	-	
BVDV2	CPAE	.	.	.	.	.	.	.	.	.	.	.	AA	UA	CC	U	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	.	-	-	
BVDV2	EBTr	.	.	.	.	.	.	.	.	.	.	.	AA	UA	CC	U	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	.	-	-	
BVDV2	HE727	GU	.	.	.	.	.	.	.	.	.	.	AA	UA	CC	U	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	.	-	-	
BVDV2	MMR-T	GU	.	.	.	.	.	.	.	.	.	.	AA	UA	CC	U	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	.	-	-	
BVDV2	Parvo	GU	.	.	.	.	.	.	.	.	.	.	AA	UA	CC	U	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	.	-	-	
BVDV2	Rubella	GU	.	.	.	.	.	.	.	.	.	.	AA	UA	CC	U	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	.	-	-	
BVDV2	V-FLL	.	.	.	.	.	.	.	.	.	.	.	UA	UG	CU	G	-	AU	UG	.	.	.	.	.	.	.	.	.	GC	.	.	.	.	.	.	CC	-	-	

Continental Europe

Position	V1	1	2	3	7	8	9	12	13	14	15	16	17	18	19	20	21	22	V2	1	2	3	4	5	6	7	8	9	V3	1	2	3	4	5	6	7	8	9	10
BVDV2	AU	UA	GC	UA	CG	GC	AU	CG	UA	CG	CG					AU				CG	CG			AU	UA	GC	CG	GU	AU	GU	CG		CG	CG	AU	UG	UC	-	
Cattle strains characterized by V3/7 AC V3/8 CA																																							
BVDV2	AF112												AA	UG	CU	G	-		AC				UA								AU		AC	CA	-				
BVDV2	B45-5												AA	UG	CU	G	-		AC				UA								AU		AC	CA	-				
BVDV2	B5-4												AA	UG	CU	G	-		AC				UA								AU		AC	CA	-				
BVDV2	B77-5					GG	GA						AA	UG	CU	G	-		AC				UA								AU		AC	CA	-				
BVDV2	BS-95-II												AA	UG	CU	G	-		AC				UA								AU		AC	CA	-				
BVDV2	Giessen-1												AA	UG	CU	G	-		AC				UA								AU		AC	CA	-				
BVDV2	Munich 3												UA	CG	CU	G	-		AC				UA								AU		AC	CA	-				
BVDV2	B50-5												AA	UG	CU	G	-		AC				UA								AU		AC	CA	-				
2a 1 1 only European																																							
BVDV2	BSE1239												AA	UG	CU	G	-		AU				UG								AU					-			
BVDV2	UVR420												AA	UG	CU	G	-		AU				UG							GC	AU					-			
Contaminant similar with UK divergent V2/5 AA																																							
BVDV2	MP												AU	UA	CC	U	-		AU				UG	AA							GC					-			
Similar with USA 2a 1 6 divergent V1/21, similar with Argentine divergent V1/1 showing GU instead of AU																																							
BVDV2	104/98												AA	UG	CU	G	-		GU				UA	GU							GC		GU			-			
BVDV2	B52-2												AA	UG	CU	G	-		GU				UA	GU							GC		GU			-			
BVDV2	Munich 1												AA	UG	CU	G	-		GU				UA	GU							GC		GU			-			
BVDV2	Munich 2												AA	UG	CU	G	-		GU				UA	GU							GC		GU			-			
BVDV2	4-5174												AA	UG	CU	G	-		GU				UA	GU							GC		GU			-			
2a 1 3 similar with UK strains divergent V1/17, 20																																							
BVDV2	BSE921												AA	UA	AC	U	-		AU				UG									GC					-		
2a 1 4 identical with North American																																							
BVDV2	IT-1732												AA	UG	UC	G	-		AU				CG	GU								GC		UG			-		
BVDV2	WG4622												AA	UG	UC	G	-		AU				CG	GU								GC		UG			-		
BVDV2	Kosice												AA	UG	UU	G	-		AU				CG									GC		UG			-		
BVDV2	37Gr												AA	UG	UU	G	-		AU				CG									GC		UG			-		
BVDV2	11/Mi/97												AA	CG	CC	G	-		AU				UG				UG					GC					-		
BVDV2	15-103												AA	UG	UC	G	-		AU				CG									GC		UG			-		
BVDV2	BSE341												AA	UG	CU	G	-		AU				UG								GC		GU				-		
BVDV2	WVD829					GG							AA	UG	CU	G	-		AU				UG								GC		GU				-		
New Zealand																																							
Position	V1	1	2	3	7	8	9	12	13	14	15	16	17	18	19	20	21	22	V2	1	2	3	4	5	6	7	8	9	V3	1	2	3	4	5	6	7	8	9	10
BVDV2	AU	UA	GC	UA	CG	GC	AU	CG	UA	CG	CG					AU				CG	CG			AU	UA	GC	CG	GU	AU	GU	CG		CG	CG	AU	UG	UC	-	
Similar with USA 2a 1 1 divergent V1/20 AC																																							
BVDV2	97/730												AA	UG	CC	AC	A	-		AU				UG								AU					-		

Fig. 1. BVDV2 palindromic secondary structure sequences and relation with geographical origin. Sequences are segregated according to geographic relevant groups. Specific characters on originality, shared sequences or variations are presented.

tal Europe, UK, New Zealand and Japan. Fied cattle strains from continental Europe, Japan and U.S.A., characterized by V3/7 AC and clustered in the same subgenotype 2 of genotype 2a, were divergent in V3/8: German isolates showed CA, Japanese strains were characterized by UG or UU, strain from U.S.A. showed UA. Genomic variation could distinguish also strains isolated from Brazil and from Argentina. Within genotype 2b, Argentinean strains were divergent from Brazilian ones at the level of the V2 locus in position 1 showing GU and AU, respectively. Argentinean strains belonging to genotypes 2b and 2d were characterized by V1/20 AA. Despite similarity within genotype 2a, subgenotype 1, variant 6, Argentinean strains showed specific nucleotide changes from other strains from Europe and U.S.A., clustered in the same variant. Argentinean cattle strains were similar with U.S.A. 2a 1 6 divergent V1/21, and similar with European divergent V1/1 showing GU instead of AU pairing. Similarly, strains affecting sheep showed divergence from those isolated from cattle. They correspond to variants 2 and 3 of subgenotype 1 within the genotype 2a. The only strain affecting cattle showing relation being included in variant 13 were divergent in position 19 of V1. Among sequences originated from sheep, those from UK were characterised by AU pairing in position 17, UA in position 18 and U in position 20 in the V1 locus. Those from U.S.A.

showed a characteristic CA bulge in position 6 of V2 locus. Contamination of biological products was related to genotypes 2a and 2d.

Correlation between clinical features related with isolation of BVDV-2 strains with genetic variation indicated that genotype 2a; subgenotype 1, variants 4 and 5 were related with hemorrhagic syndrome, and apparently as exception also variant 6 (Table 3). Within strains affecting cattle, highly virulent ones, exception made for 2 strains (AZ Spl and CD87), they were characterised by V1/19 UU or UC and V2/4 CG (stronger binding than UG detected in all low virulent strains). Similarly, ovine isolates from U.S.A. showed the same characteristic. Ovine strains related with border disease from UK showed characteristic UA pairing in V1/18 and U in V1/21, shared by only one low virulent cattle strain. Low virulent cattle strains sequences showed in V1/19 CC, CU, AC, CA bulges or CG pairing, and in V2/4 UG or UA.

The genotype BVDV-2a subgenotype 1 variant 2, 4 and 5 strains, including the hyper-virulent strain 890 (variant 5), presented mainly uracil as N1 and cytosine as N2 (UC), and all were related to high virulence (hemorrhagic syndrome or acute BVD). Within variant 4, six strains presented the CU combination, but only one was related with high virulence as hemorrhagic syndrome. In the genotype BVDV-2a sub-

Table 3. Summary of correlation between clinical features related with isolation of BVDV-2 strains with genetic variation

Highly virulent strains						
Genotype	Subgenotype	Variant	Host	Clinical presentation	Number of observations	
BVDV-2a	1	2	Sheep	Border disease	2	
BVDV-2a	1	3	Sheep	Border disease	5	
BVDV-2a	1	4	Cattle	Hemorrhagic syndrome	11	
BVDV-2a	1	5	Cattle	Hemorrhagic syndrome	1	
BVDV-2a	1	6	Cattle	Hemorrhagic syndrome	1	
Mucosal disease						
BVDV-2a	2	–	Cattle	Fatal mucosal disease	1	
BVDV-2b	2	–	Cattle	Mucosal disease	1	
Low virulent strains						
Genotype	Subgenotype	Variant	Host	Clinical presentation	Number of observations	
BVDV-2a	1	1	Cattle	Fetal abortion	5	
BVDV-2a	1	3	Cattle	Neurological symptoms	1	
BVDV-2a	1	4	Cattle	Neurological symptoms	3	
BVDV-2a	1	6	Cattle	Mild symptoms	7	
BVDV-2a	2	–	Cattle	Respiratory symptoms, abortion	12	
BVDV-2b	1	1, 2, 3, 4	Cattle	Mild symptoms	6	
BVDV-2c	–	–	Cattle	Mild symptoms	1	

genotype 1 variants 1 and 6 was evident only the combination of cytosine as N1 and uracil as N2 (CU) which was associated mainly low virulence as abortion, respiratory or neurological symptoms or persistent infection, with no lethal issue, exception made in strain CD87, associated to hemorrhagic syndrome. Similarly, in genotype BVDV-2a subgenotype 2, CU was generally associated with mild clinical symptoms, apart for strain Giessen-1, associated fatal mucosal disease. The genotype BVDV-2a subgenotype 1 variant 3 presented cytosine as N1 and uracil as N2 (CU) in relation with high virulence as border disease in sheep isolates as well as with mild clinical symptoms in one cattle isolate, and in contaminants. Furthermore, one BVDV-2a strain presented adenine as N1 and uracil as N2 (AU) related with low virulence as neurological symptoms. South American isolates belonging to genotypes BVDV2-b, BVDV2-c and BVDV2-d showed CU combination in relation with mild clinical course or contamination (genotype 2c), apart subgenotype 2 of BVDV2-b related with MD.

In strains isolated from sheep, all animals with symptoms of border disease, the presence of cytosine in position N1 and uracil in position N2, was the most frequent, in 5 out of 7 strains, Lees, 167 237, 168 149, 173 157 and 175 375 (all strains originated from UK). The presence of uracil in position N1 and cytosine in position N2 was observed in the two North American strains BD-78 and C413. In BVDV-2 strains adventitious contaminants of biological products, the presence of uracil in position N1 and cytosine in position N2 was observed in only two strains, the high virulence related strains WG4622 and IT-1732. All other tested strains, cell lines and biological products contaminants, with unknown virulence degree, showed cytosine in position N1 and uracil

in position N2.

DISCUSSION

The observation made on the nucleotide sequences of the three variable loci at the level of the 5'-UTR genomic region of BVDV-2 *Pestivirus* strains allowed to the identification of consensus motifs shared by all the *Pestivirus* species. The BVDV-2 strains could be clearly differentiated genetically, in terms of specific nucleotide substitutions, by the BVDV1 species. Four genotypes, BVDV-2a, BVDV-2b, BVDV-2c, and BVDV-2d, were classified within BVDV-2 by the PNS method. South American isolates represented three well distinct clusters among strains in the species. Relevant nucleotide variations in the secondary structure were evident at the level of the V1 palindromic locus. Genotypes BVDV-2c and BVDV-2d were restricted to Argentina. BVDV-2b was present in Brazil and Argentina, suggesting a link with animal exchange, even if the observation was made on a very limited number of available strains. However, between 1980 and 2002, limited bovine imports from Brasil were recorded (1 breeder in 1986, 1 breeder in 1988, 2 breeders in 1989, 1 breeder in 1990, 326 fattening cattle in 1998 and 211 in 1999) (National Animal Health Service, Buenos Aires, Argentina). Importation of bubalines has been also recorded (154 in 1998, 432 in 1999), but no sheep imports. In the same period, the number animals imported from Uruguay was higher (around 150,000 heads for slaughtering, and 5,000 breeders). Despite absence of epidemiological data, this could suggest an eventual indirect source of virus diffusion in the region through a neighbouring country which is geographically situated between Brazil and

Argentina.

Nomenclature for Pestivirus species is predominantly dependent on the animal host species from which they were isolated. There is extensive antigenic cross-reactivity among the species, and, they can cross the host species barrier and infect various animal species. The application of genetic analyses appeared necessary as most appropriate approach for differential diagnostic in order to solve such cross-infections which may obscure the rationale for the definition of the *Pestivirus* species according to their animal host. In the present study, eight BVDV-2 strains were ovine isolates. The sequence analysis showed that only BVDV-2a was related to virus circulation in sheep, whereas genotypes BVDV-2c, and BVDV-2d were prevalent in cattle. Strains have been isolated from animals suffering of border disease, similar symptoms caused with BDV, thus rising confusion in diagnosis. Among BDV strains previously classified depending on the host animal species, occurrence of other *Pestivirus* species has long been suspected [7, 10, 29]. Technical terms "true" BDV and "atypical" BDV have been used in order to discriminate authentic BDV strains from false BDV strains by Thiel and his colleagues [1, 3, 4]. This confusion was further examined by phylogenetic analysis of the E2 (gp53) region, Npro gene or 5'-UTR of ovine pestiviruses, and the BDV strains previously classified have been shown to include BVDV [3, 34, 38]. In a recent study, by using the PNS genotyping procedure, ovine isolates showed palindromic structure in the 5'-UTR characteristic to the BVDV-1a and BVDV-1b genotypes, and to BVDV-2 [15]. The number of BVDV-2 ovine isolates, reported until now, is limited. In addition, it is not excluded that the five ovine strains reported by Vilcek *et al.* [38] are contaminants from fetal calf serum during cultivation of cells [37]. Therefore, particular attention is required in performing isolation on cell. RT-PCR assay for the rapid recognition of virus in blood sample or original tissue homogenates with no cultivation on cells for border disease clinical cases could be a useful approach.

The two nucleotides, related to virulence degree in Pestivirus strains, were identifiable at the palindromic structures, variable loci V1 loop and V2 stem regions in the IRES in the 5'-UTR of the genomic RNA. Different nucleotide combinations could be identified. The most frequent was CU. UC and AU pairings were less frequent. The two nucleotide combinations UC and CU resulted associated to high virulence and in relation with genotype BVDV-2a. The nucleotide combination CU was shared with low virulence strains and in relation to genotypes BVDV-2a, 2b and 2c. In cattle, the presence of uracil in position N1 and cytosine in position N2 was observed in 11 strains related to severe clinical disease. The presence of cytosine in position N1 and uracil in position N2 was observed in 39 strains, exception made for 2 strains, mainly related to mild forms of clinical disease. The presence of cytosine at position N1 and uracil at position N2 was identified in two field isolates related to hemorrhagic syndrome from North America. In one strain was observed adenine in position N1 and uracil in

position N2. The observation made by Topliff and Kelling [35] could be partially confirmed in our study. Out of the 13 tested cattle strains related to high virulence, the 84.61% showed the suggested virulence marker UC. However, the strains 890 and CD87 had to be considered of high virulence and low virulence, respectively, giving that the strain 890 presented uracil in position N1 and cytosine in position N2, and the strain CD87 showed the 2 nucleotides in the inverted locations, with cytosine at position N1 and uracil at position N2. Both strains were reported by Pellerin *et al.* [25] from outbreaks in Canadian cattle, thus, equally responsible of haemorrhagic syndrome. Similarly, the strain AZ Spl, responsible of haemorrhagic syndrome in U.S.A., both showed N1 C and N2 U, the alleged low virulence related nucleotide combination.

In sheep, BVDV-2 was responsible for severe illness irrespective for the presence of UC or CU combination, indicating the absence of direct relationship with clinical manifestation of Border disease. However, the presence of uracil in position N1 and cytosine in position N2 was observed in the 2 North American isolates. Cytosine in position N1 and uracil in position N2 was detected in the five strains from UK, which have been suspected contaminants [38].

It seems appropriate to refer to the suggested nucleotide combination as virulence marker. In the case of the iatrogenic accident occurred in the Netherlands among cattle, who received a live virus vaccine contaminated with pestivirus BVDV-2, genetic investigations performed by State Institutes indicated two specific nucleotide substitutions in the 5'-UTR as characteristic of highly virulent BVDV-2, according to Topliff and Kelling [11]. However, this could raise the risk of underestimation for other nucleotide combinations potentially related to high virulence.

The observation of Pilipenko on the poliovirus 5'-UTR demonstrated important aspects; useful base for investigations on virulence and the study of Topliff and Kelling revealed similar relationship between virulence characteristic and the IRES of BVDV-2. However, increasing the number of evaluated strains, it appeared that the virulence nucleotide markers are more complex and the definition of virulence activity among BVDV-2 species, in terms of nucleotide sequence, remains still undefined. Furthermore, it is unclear the high variability in the other *Pestivirus* species, despite the genomic similarity to poliovirus, as for BVDV-2. In conclusion, uracil in position N1 and cytosine in position N2 appeared to be related to high virulence in the BVDV-2 species, but it was not always determinative for virulence markers since high virulence was also associated to cytosine in positions N1 and N2 and cytosine in position N1 and uracil in position N2.

The palindromic loci represented, with 77–85 nucleotides, a very limited portion of the virus genome. Within these short sequences, it was sufficient the evaluation of only 18 nucleotides to obtain with certitude the characterization of the genus. Species were characterized through the evaluation of only 2 to 8 nucleotides. Similarly, the geno-

type was defined with only 2 to 6 nucleotides. These peculiar aspects resumed the high specificity of the PNS method and the reliability of the provided results. The palindromic nucleotide substitution analysis in the 5'-UTR demonstrated a rationale and simple approach for viral investigations. Secondary structures predicted at the three variable regions in the 5'-UTR showed typical PNS which were useful for classification or genotyping of *Pestivirus*. Due to the worldwide economic importance of these viruses, and the difficulties encountered in the control of the diseases, it is, therefore, important to understand the genetic aspects of the viruses and their evolutionary history. These observations suggest that evaluation of genomic secondary structure, by identifying markers of virus genomic characteristics in relation with environment—geographic segregation as well as animal host—and clinical course, may be applied as useful tool for epidemiological evaluation, improving our understanding of the BVDV-2 species epizootiology, and possibly for other species of the genus *Pestivirus*.

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