Taxonomic and epidemiological aspects of the bovine viral diarrhoea virus 2 species through the observation of the secondary structures in the 5′ genomic untranslated region

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Summary

Bovine viral diarrhoea virus 2 (BVDV-2) strains demonstrated in cattle, sheep and adventitious contaminants of biological products were 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 taxonomic status. Variation in conserved genomic sequences was used as a parameter for the epidemiological evaluation of the species in relation to geographic distribution, animal host and virulence. Four genotypes were identified within the species. Taxonomic segregation corresponded to geographic distribution of genotype variants. Genotype 2a was distributed worldwide and was also the only genotype that was circulating in sheep and cattle. Genotypes 2b, 2c and 2d were restricted to South America. Genotypes 2a and 2d were related to the contamination of biological products. Genetic variation could be related to the spread of BVDV-2 species variants in different geographic areas. Chronologically, the species emerged in North America in 1978 and spread to the United Kingdom 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 subgenotype 1, variant 4 of genotype 2a, was related to a haemorrhagic syndrome. These observations suggest that the evaluation of genomic secondary structures, by identifying markers for expression of virus biological activities and species evolutionary history, may be a useful tool for the epidemiological evaluation of BVDV-2 species and possibly of other species of the genus *Pestivirus*.

Keywords

Bovine viral diarrhoea, BVDV-2, Palindromic nucleotide substitutions, Pestivirus, 5′-untranslated region, Virus.

Aspetti tassonomici ed epidemiologicici della specie del virus della diarrea virale bovina tipo 2 attraverso l'osservazione delle strutture secondarie nella regione genomica non tradotta 5′

Riassunto

Ceppi del virus della diarrea virale bovina 2 (BVDV-2) isolati in bovini, pecore e contaminanti

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avventizi di prodotti biologici sono stati valutati con il metodo delle sostituzioni nucleotidiche palindromiche (PNS) a livello dei tre siti variabili (V1, V2 e V3) nella regione non tradotta (UTR) 5′, per determinare il loro stato tassonomico. Le variazioni in sequenze genomiche conservate sono state usate come parametro per la valutazione epidemiologica della specie sulla distribuzione geografica, animali ospiti e virulenza. Quattro genotipi sono stati identificati nella specie. La segregazione tassonomica ha corrisposto alla distribuzione geografica delle varianti dei genotipi. Il genotipo 2a ha mostrato una distribuzione cosmopolita ed era l'unico genotipo responsabile di infezione sia in pecore che bovini. I genotipi 2b, 2c e 2d erano limitati al Sud America. I genotipi 2a e 2d erano correlati alla contaminazione di prodotti biologici. La variazione genetica ha potuto essere correlata alla diffusione delle varianti della specie BVDV-2 in differenti aree geografiche. Cronologicamente, la specie è emersa in Nord America nel 1978 e si è diffusa nel Regno Unito e Giappone, Europa continentale, Sud America e Nuova Zelanda. Correlazioni tra quadri clinici riferiti ad isolamento di ceppi di BVDV-2 e variazioni genetiche hanno indicato che il sottogenotipo 1, variante 4 del genotipo 2a, era in relazione ad una sindrome emorragica. Queste osservazioni suggeriscono che la valutazione della struttura genomica secondaria, identificando marcatori di espressione di attività biologiche virali e di storia evolutiva della specie, potrebbe essere applicata come utile mezzo per la valutazione epidemiologica della specie BVDV-2 e possibilmente per altre specie del genere Pestivirus.

Parole chiave

Diarrea virale bovina, BVDV-2, Pestivirus, Sustituzioni nucleotidiche palindromiche, 5′-UTR, Virus.

Introduction

Bovine viral diarrhoea virus 2 (BVDV-2) is an established species of the genus *Pestivirus*, family *Flaviviridae*, with bovine viral diarrhoea virus-1 (BVDV-1), Border disease virus (BDV) and classical swine fever virus (CSFV) and a tentative 'giraffe' species (16). BVDV-2 species includes the strains isolated from outbreaks of haemorrhagic syndrome characterised by thrombocytopenia and high mortality in the United States and Canada (7, 37, 39). Hypervirulent strains 890 and CD-87 were previously included in BVDV species in a separate cluster (37, 40). The haemorrhagic syndrome has also been reported in European cattle (10, 30, 45). Different strains were also isolated in bovines presenting mild clinical forms (33, 47). Furthermore, the presence of the species was observed in biological products as adventitious contaminants (1, 21, 21, 23), indicating the risk of iatrogenic infection demonstrated by the recent occurrence of a serious accident in the Netherlands among cattle that received a live virus vaccine contaminated with *Pestivirus* (15).

The *Pestivirus* genome, a single-stranded, positive polarity RNA, is composed of 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 conserved among all members within the genus *Pestivirus*, and thus is useful for the characterisation of species or genotypes. Primary structure analysis, using by sequence alignment and construction of phylogenetic trees, is the most common method for the classification of *Pestivirus* isolates. 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 (13, 21), a critical region of the 5′-UTR which is responsible for translational, transcriptional and replication 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 significant importance in terms of the history of evolution of the virus. The nucleotide substitutions of the IRES in the 5′-UTR were reportedly responsible for the

virulence of poliovirus (38). Mutations or nucleotide substitutions at palindromic IRES regions will affect efficiency translation from RNA to protein. Thus, low translation reduces virulence and high translation increases virulence. Due to the similarity of genetic structure among pestiviruses, this concept was proposed for BVDV-2 by Topliff and Kelling (47). The authors reported a similarity between high virulence and two specific nucleotides (uracil and cytosine) located at positions 219 and 278 of the genomic sequence (based on BVDV-1 strain Osloss) and low virulence (with cytosine and uracil) at positions 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 specific to each *Pestivirus* species. Nucleotide substitutions in the stem regions always occur and maintain the palindromic sequence, thereby forming a stable stem-loop structure. Thus, this type of mutation was referred to as palindromic nucleotide substitutions (PNS). Based on the above considerations, the observation of nucleotide variations among virus strains at the level of the three specific palindromes in the 5′-UTR has been considered as a genotyping method (26). PNS analysis appeared to be simple and practical, showing comparable results with other procedures based on 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 assist in the classification species and genotype boundaries, due to the exclusive consideration of strategic and highly conserved regions, and consequently would avoid unclear classification.

In our study, BVDV-2 *Pestivirus* strains, isolates from cattle and sheep and adventitious contaminants of biological products, were evaluated with PNS analysis in the 5′-UTR to determine the relationship between genotypic variations and epidemiological observations.

Materials and methods

The nucleotide sequences in the 5′-UTR of 73 BVDV-2 species strains were obtained from the DNA databases or provided by the authors (Table I). According to the geographic origin of the virus isolates, 15 were reported from North America and 12 from South America, 32 from Europe, 13 from Japan and 1 from New Zealand. A total of 8 strains originated from sheep, isolated from outbreaks of Border disease, 12 strains were detected as being contaminants from cell cultures and biologicals. Strain IT-1732 (AJ416018) (M.M. Muscillo, unpublished findings) was isolated from a bayovac IBR-marker vaccine (Bayer), responsible for outbreaks in The Netherlands and Italy (15). Strain WG4622 (ALIGN_000012) (12) was isolated from the same batch of vaccine. Strains MMR-T (D26052), Rubella (D26048) (21, 23) and HE727 (D31807) (25), were isolated from Japanese commercial virus vaccines and interferons for human use. Strain 354 (AF244959) (28) was isolated in Argentina.

The secondary structures were predicted according to the algorithm of Zuker and Stiegler (55). The minimum free energy was calculated using the method of Freier *et al.* (18). Three variable regions, V1, V2 and V3, at the 5′-UTR were used for genotyping based on palindromic nucleotide substitutions (26). Sequences were aligned according to secondary structure to evaluate nucleotide changes in relation with geographic distribution, animal host and virulence, identifying specific PNS markers.

Results

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

Table I
Bovine viral diarrhoea 2 strains evaluated according to secondary structure nucleotide changes

Genotypes	Strain	Origin	Country	Year of isolation	Clinical presentation	Accession No.	Ref.
BVDV-2a1.1	5521-95	Cattle	United States	1995	Foetal abortion	AF039174	47
BVDV-2a1.1	713-2	Cattle	United States	1982	Foetal abortion	AF039177	47
BVDV-2a1.1	97/730	Cattle	New Zealand	1997	Not available	AF026770	Vilcek *et al.*[a]
BVDV-2a1.1	BSE1239	Cattle	Belgium	1996	Neurological symptoms	ALIGN_000012	12
BVDV-2a1.1	UVR420	Cattle	Belgium	1991	Calf intestine	ALIGN_000012	12
BVDV-2a1.2	BD-78	Sheep	United States	1978	Border disease	U18330	43
BVDV-2a1.2	C413	Sheep	United States	2002	Border disease	AF002227	Chen & Berry[a]
BVDV-2a1.3	167 237	Sheep	United Kingdom	1987	Border disease	U65055	49
BVDV-2a1.3	168 149	Sheep	United Kingdom	1987	Border disease	U65056	49
BVDV-2a1.3	173 157	Sheep	United Kingdom	1987	Border disease	U65058	49
BVDV-2a1.3	175 375	Sheep	United Kingdom	1987	Border disease	U65059	49
BVDV-2a1.3	BSE921	Cattle	Belgium	1995	Neurological symptoms	ALIGN_000012	12
BVDV-2a1.3	Lees	Sheep	United Kingdom	1985	Border disease	U65051	49
BVDV-2a1.3	CPA	Contam[b]	Japan	1993	Not applicable	D50812	24
BVDV-2a1.3	CPAE	Contam[b]	Japan	1993	Not applicable	D50813	24
BVDV-2a1.3	EBTr	Contam[b]	Japan	1993	Not applicable	D50817	21
BVDV-2a1.3	HE727	Contam[b]	Japan	1995	Not applicable	D31807	24
BVDV-2a1.3	MMR-T	Contam[b]	Japan	1993	Not applicable	D26052	23
BVDV-2a1.3	MP	Contam[b]	Belgium	2002	Not applicable	ALIGN_000012	12
BVDV-2a1.3	Parvo	Contam[b]	Japan	1994	Not applicable	D26614	21
BVDV-2a1.3	Rubella	Contam[b]	Japan	1993	Not applicable	D26048	23
BVDV-2a1.4	11/Mi/97	Cattle	Italy	1997	Not available	AJ293603	33
BVDV-2a1.4	15-103	Cattle	France	1994	Not available	AF298055	51
BVDV-2a1.4	17583-97	Cattle	United States	1997	Acute BVD	AF039176	47
BVDV-2a1.4	23025	Cattle	United States	1993	Haemorrhagic syndrome	AF039172	47
BVDV-2a1.4	37Gr	Cattle	Austria	1998-2000	Acute diarrhoea	Not deposited	53
BVDV-2a1.4	7937	Cattle	United States	1988	Persistent infection	AF039175	47
BVDV-2a1.4	AZ Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome	Not deposited	40
BVDV-2a1.4	BSE341	Cattle	Belgium	1991	Neurological symptoms	ALIGN_000012	12
BVDV-2a1.4	IT-1732	Contam[b]	Italy	1999	Acute BVD	AJ416018	Muscillo[a]
BVDV-2a1.4	Kosice	Cattle	Slovakia	2002	Fatal course, malformations, haemorrhages	Not deposited	52

Table I (contd)
Bovine viral diarrhoea 2 strains evaluated according to secondary structure nucleotide changes

Genotypes	Strain	Origin	Country	Year of isolation	Clinical presentation	Accession No.	Ref.
BVDV-2a1.4	MAD Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome	Not deposited	40
BVDV-2a1.4	MN Foetus	Cattle	United States	1960-1993	Haemorrhagic syndrome	Not deposited	40
BVDV-2a1.4	NY93	Cattle	United States	1993	Acute BDV	AF039173	47
BVDV-2a1.4	Q126	Cattle	Canada	1994	Haemorrhagic syndrome	L32890	37
BVDV-2a1.4	V-FLL	Contam(b)	Japan	1999	Not applicable	AB019687	42
BVDV-2a1.4	WG4622	Contam(b)	Netherlands	1999	Lethal acute BVD	ALIGN_000012	12
BVDV-2a1.4	WVD829	Cattle	Belgium	1994	Respiratory symptoms	ALIGN_000012	12
BVDV-2a1.5	890	Cattle	Canada	1990	Haemorrhagic syndrome	L32886	37
BVDV-2a1.6	104/98	Cattle	Germany	1998	Mild symptoms	AJ304381	44
BVDV-2a1.6	B52-2	Cattle	Germany	92-99	Mild symptoms	Not deposited	5
BVDV-2a1.6	CD87	Cattle	Canada	1987	Haemorrhagic syndrome	L32887	37
BVDV-2a1.6	i4083	Cattle	Argentina	2001	Mild symptoms	AF417995	Jones *et al.*(a)
BVDV-2a1.6	i61380	Cattle	Argentina	2001	Mild symptoms	AF417986	Jones *et al.*(a)
BVDV-2a1.6	i628	Cattle	Argentina	2001	Mild symptoms	AF417985	Jones *et al.*(a)
BVDV-2a1.6	Munich 1	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a1.6	Munich 2	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a1.6	4-5174	Cattle	France	1994	Not available	AF298063	51
BVDV-2a2	B45-5	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a2	B50-5	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a2	B5-4	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a2	B77-5	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a2	Giessen-1	Cattle	Germany	1996	Fatal mucosal disease	AF104030	4
BVDV-2a2	Munich 3	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a2	17011-96	Cattle	USA	1996	Foetal abortion	AF039179	47
BVDV-2a2	AF112	Cattle	Germany	1992-1999	Mild symptoms	Not deposited	5
BVDV-2a2	BS-95-II	Cattle	Italy	1995	Not available	AJ288903	33
BVDV-2a2	MS-1	Cattle	Japan	1999	Persistent infection	AB019688	42
BVDV-2a2	SW90	Cattle	Japan	1990	Mild symptoms	AB003622	35
BVDV-2a2	SY-89	Cattle	Japan	1989	Persistent infection	AB019689	42

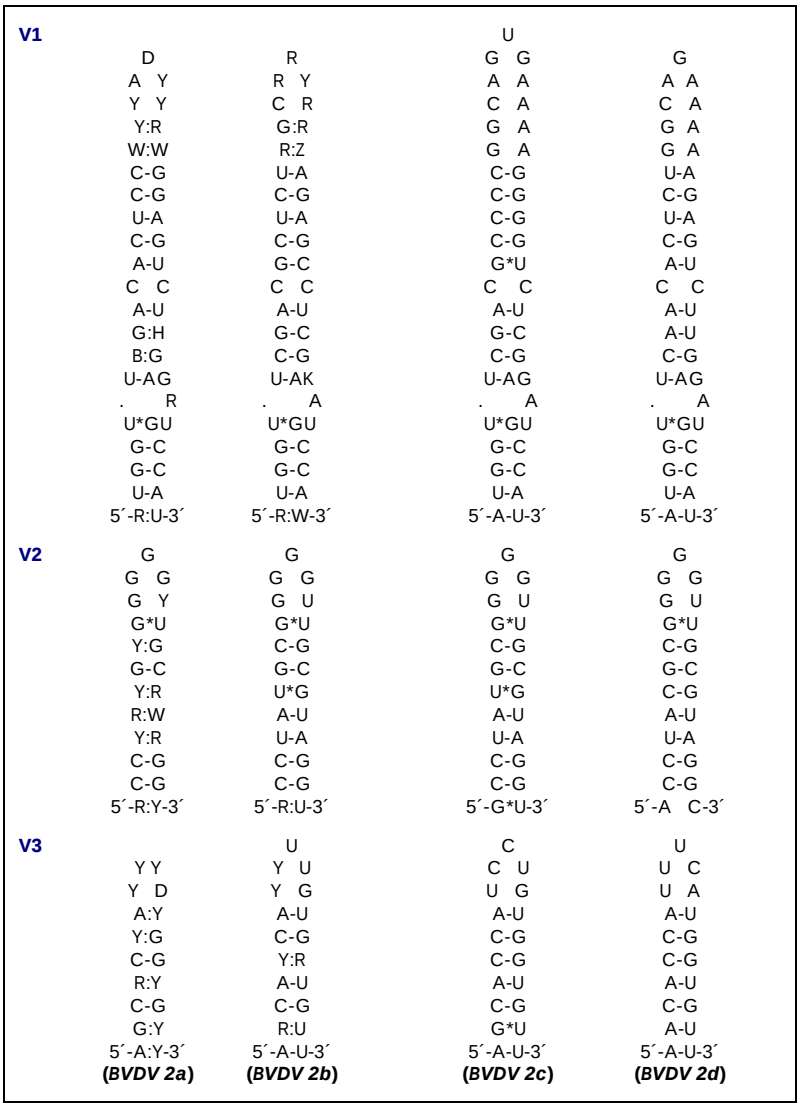
Table I (contd)
Bovine viral diarrhoea 2 strains evaluated according to secondary structure nucleotide changes

Genotypes	Strain	Origin	Country	Year of isolation	Clinical presentation	Accession No.	Ref.
BVDV-2a2	OY89	Cattle	Japan	1989	Persistent infection, respiratory symptoms	AB003621	34
BVDV-2a2	TC Shinozaki	Cattle	Japan	1992	Persistent infection, respiratory symptoms	AB04267	35
BVDV-2b1.1	VS-63	Cattle	Brazil	1996	Healthy foetus	AF410789	17
BVDV-2b1.2	34b	Cattle	Argentina	1991	Foetus	AF244952	28
BVDV-2b1.2	ncp7	Cattle	Argentina	1993	Foetus	AY443026	28
BVDV-2b1.3	VS-123.4	Cattle	Brazil	1996	Healthy foetus	AF410790	17
BVDV-2b1.4	LV-96	Cattle	Brazil	1996	Chronic gastroenteric symptoms	AF410787	17
BVDV-2b1.4	VS-260	Cattle	Brazil	1997	Gastroenteric symptoms	AF410788	17
BVDV-2b2	Soldan	Cattle	Brazil	1991	Mucosal disease	U94914	Canal *et al.*(a)
BVDV-2c	i33283	Cattle	Argentina	2001	Mild symptoms	AF417996	Jones *et al.*(a)
BVDV-2d	354	Contam(b)	Argentina	1995	Not applicable	AF244959	28
BVDV-2(c)	59386	Sheep	United Kingdom	1994	Border disease	U17146	1
BVDV-2(c)	SCP	Contam(b)	United Kingdom	1994	Not applicable	U17148	1

(a) unpublished
(b) contaminant
(c) not determined

Characteristic base pairings common to BVDV-2 species were observed in all isolates tested. The application of the PNS method showed four genotypes, BVDV-2a, BVDV-2b, 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 their South American counterparts. Genotype 2b, 2c and 2d strains from South America were characterised by V1/17 GA or GG or AC V1/18 GG or GA and a higher V3 loop (Fig. 1). Genotype 2c shared characteristic pairing GC in V1/12 with 2b. Thus V1/16 showed CG common pairing in strains from other continents, instead of UA pairing common with other South American strains. In addition, the specific CG pairing in position 14 of V1 characterised genotype 2c. Genotype 2d shared UA pairing in V1/16 with 2b, 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 genotype 2d. Genotype 2a was present worldwide and was the only genotype found to be circulating in sheep and cattle, the prevalent host species. Genotypes 2b, 2c and 2d were restricted to South America (Tables II and III).

Taxonomic segregation corresponded to geographic distribution of genotype variants. Chronologically, the species was first detected in North America in 1978 and spreading to the United Kingdom and Japan, and later in continental Europe, South America and New Zealand (Tables IV and V). Genetic variation could be correlated to chronological spread of the BVDV-2 species variants in different geographic areas (Tables VI and VII; Figs 2 and 3).



- Watson-Crick base pairings
* tolerated pairings in secondary structure
: interchangeable base pairings

M= A or C; R= A or G; W= A or U; S= C or G; Y= C or U; K= G or U; Z= A or C or G; H= A or C or U; D= A or G or U; B= C or G or U; N= A or C or G or U

Figure I
Secondary structure of bovine viral diarrhoea virus 2 genotypes: V1, V2 and V3 palindromic loci in the 5′-UTR

Genotype 2a subgenotype 1 variant 1, 4 and 6, first reported in North America in 1982 and again in 1988 and 1987, has appeared to have circulated worldwide. Variant 1 was reisolated in continental Europe in 1991 and six years later in New Zealand in 1997. Variant 4, of particularly high virulence in cattle, was reported in Europe in 1991 showing mild clinical symptoms and, in 1998-2000 was associated with cases of high virulence. The variant was reported in Japan as a contaminant in 1999. Variant 6, related to haemorrhagic syndrome in Canada, was reisolated in Germany in 1994 and in Argentina in 2001 and was associated with mild symptoms. Interestingly, subgenotype 2 was first reported in Japanese cattle and reisolated in 1995 in Europe and in 1996 in the United States. Despite sequence similarities and identities generated by animal trade, specific nucleotide changes, especially at the level of V1 and V3 loops, enabled the differentiation of strains that had originated from North and South America, continental Europe, the United

Table II
Geographic distribution and origin of isolation of bovine virus diarrhoea virus 2 genotypes

Genotypes	Cattle	Sheep	Contaminant	North America	South America	Europe	Asia	Oceania
BVDV-2a	Yes	Yes	Yes	Canada, United States	Argentina	Austria, Belgium, France, Germany, Italy, Netherlands, Slovakia, United Kingdom	Japan	New Zealand
BVDV-2b	Yes	No	No		Argentina, Brazil			
BVDV-2c	Yes	No	No		Argentina			
BVDV-2d	No	No	Yes		Argentina			

Table III
Geographic distribution and origin of isolation of bovine virus diarrhoea virus 2 genotypes, subgenotypes and variants

Genotype	Subgenotype	Variant	Cattle	Sheep	Contaminant	North America	South America	Europe	Asia	Oceania
BVDV-2a	1	1	Yes	No	No	United States		Belgium		New Zealand
BVDV-2a	1	2	No	Yes	No	United States				
BVDV-2a	1	3	Yes	Yes	Yes			Belgium, United Kingdom	Japan*	
BVDV-2a	1	4	Yes	No	Yes	Canada, United States		Austria, Belgium, France, Italy, Netherlands*, Slovakia	Japan	
BVDV-2a	1	5	Yes	No	No	Canada				
BVDV-2a	1	6	Yes	No	No	Canada	Argentina	France, Germany		
BVDV-2a	2	–	Yes	No	No	United States		Germany, Italy	Japan	
BVDV-2b	1	1	Yes	No	No		Brazil			
BVDV-2b	1	2	Yes	No	No		Argentina			
BVDV-2b	1	3	Yes	No	No		Brazil			
BVDV-2b	2	–	Yes	No	No		Brazil			
BVDV-2c	–	–	Yes	No	No		Argentina			
BVDV-2d	–	–	No	No	Yes		Argentina*			

* contaminants only

Table IV
Chronology of the bovine virus diarrhoea virus 2 species

Genotype	Strain	Origin	Country	Year of isolation	Clinical presentation
BVDV-2a	BD-78	Sheep	United States	1978	Border disease
BVDV-2a	713-2	Cattle	United States	1982	Foetal abortion
BVDV-2a	Lees	Sheep	United Kingdom	1985	Border disease
BVDV-2a	CD87	Cattle	Canada	1987	Haemorrhagic syndrome
BVDV-2a	167 237	Sheep	United Kingdom	1987	Border disease
BVDV-2a	168 149	Sheep	United Kingdom	1987	Border disease
BVDV-2a	173 157	Sheep	United Kingdom	1987	Border disease
BVDV-2a	175 375	Sheep	United Kingdom	1987	Border disease
BVDV-2a	7937	Cattle	United States	1988	Persistent infection
BVDV-2a	SY-89	Cattle	Japan	1989	Persistent infection
BVDV-2a	OY89	Cattle	Japan	1989	Persistent infection, respiratory symptoms
BVDV-2a	890	Cattle	Canada	1990	Haemorrhagic syndrome
BVDV-2a	SW90	Cattle	Japan	1990	Mild symptoms
BVDV-2a	BSE341	Cattle	Belgium	1991	Neurological symptoms
BVDV-2b	34b	Cattle	Argentina	1991	Foetus
BVDV-2a	UVR420	Cattle	Belgium	1991	Calf intestine
BVDV-2b	Soldan	Cattle	Brazil	1991	Mucosal disease
BVDV-2a	TC Shinozaki	Cattle	Japan	1992	Persistent infection, respiratory symptoms
BVDV-2a	AZ Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	MAD Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	MN foetus	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	23025	Cattle	United States	1993	Haemorrhagic syndrome
BVDV-2a	NY93	Cattle	United States	1993	Acute BDV
BVDV-2b	ncp7	Cattle	Argentina	1993	Foetus
BVDV-2a	CPA	Contaminant	Japan	1993	Not applicable
BVDV-2a	CPAE	Contaminant	Japan	1993	Not applicable
BVDV-2a	EBTr	Contaminant	Japan	1993	Not applicable
BVDV-2a	MMR-T	Contaminant	Japan	1993	Not applicable
BVDV-2a	Rubella	Contaminant	Japan	1993	Not applicable
BVDV-2a	Q126	Cattle	Canada	1994	Haemorrhagic syndrome
BVDV-2a	Parvo	Contaminant	Japan	1994	Not applicable
BVDV-2a	15-103	Cattle	France	1994	Not available
BVDV-2a	WVD829	Cattle	Belgium	1994	Respiratory symptoms
BVDV-2a	4-5174	Cattle	France	1994	Not available
BVDV-2[c]	59386	Sheep	United Kingdom	1994	Border disease
BVDV-2[c]	SCP	Contaminant	United Kingdom	1994	Not applicable
BVDV-2d	354	Contaminant	Argentina	1995	Not applicable
BVDV-2a	BS-95-II	Cattle	Italy	1995	Not available
BVDV-2a	5521-95	Cattle	United States	1995	Foetal abortion
BVDV-2a	BSE921	Cattle	Belgium	1995	Neurological symptoms
BVDV-2a	HE727	Contaminant	Japan	1995	Not applicable
BVDV-2a	BSE1239	Cattle	Belgium	1996	Neurological symptoms
BVDV-2a	Giessen-1	Cattle	Germany	1996	Fatal mucosal disease
BVDV-2a	17011-96	Cattle	United States	1996	Foetal abortion

Table IV (contd)
Chronology of the bovine virus diarrhoea virus 2 species

Genotype	Strain	Origin	Country	Year of isolation	Clinical presentation
BVDV-2b	VS-63	Cattle	Brazil	1996	Healthy foetus
BVDV-2b	VS-123.4	Cattle	Brazil	1996	Healthy foetus
BVDV-2b	LV-96	Cattle	Brazil	1996	Chronic gastroenteric
BVDV-2a	11/Mi/97	Cattle	Italy	1997	Not available
BVDV-2a	97/730	Cattle	New Zealand	1997	Not available
BVDV-2b	VS-260	Cattle	Brazil	1997	Gastroenteric symptoms
BVDV-2a	17583-97	Cattle	United States	1997	Acute BVD
BVDV-2a	104/98	Cattle	Germany	1998	Mild symptoms
BVDV-2a	37Gr	Cattle	Austria	1998-2000	Acute diarrhoea
BVDV-2a	B52-2	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	Munich 1	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	Munich 2	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	B45-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	B50-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	B5-4	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	B77-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	Munich 3	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	AF112	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	IT-1732	Contaminant	Italy	1999	Acute BVD
BVDV-2a	V-FLL	Contaminant	Japan	1999	Not applicable
BVDV-2a	WG4622	Contaminant	Netherlands	1999	Lethal acute BVD
BVDV-2a	MS-1	Cattle	Japan	1999	Persistent infection
BVDV-2c	i33283	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	i4083	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	i61380	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	i628	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	MP	Contaminant	Belgium	2002	Not applicable
BVDV-2a	Kosice	Cattle	Slovakia	2002	Fatal course, malformations, haemorrhages
BVDV-2a	C413	Sheep	United States	2002	Border disease

Table V
Observations on chronological distribution of bovine virus diarrhoea virus 2 species

Year	Observations
1978	First report of highly virulent BVDV-2 in sheep in North America
1982	First report of low virulent BVDV-2 in cattle in North America
1985	First report of highly virulent BVDV-2 in sheep in UK
1987	First report of highly virulent BVDV-2 in cattle in North America
1989	First report of low virulent BVDV-2 in cattle in Japan
1991	First report of low virulent BVDV-2 in cattle in Continental Europe
1991	First report of low virulent BVDV-2 in cattle in South America
1996	First report of highly virulent BVDV-2 in cattle in Continental Europe
1997	First report of low virulent BVDV-2 in cattle in New Zealand

Note: In North America, highly virulent strains circulated in parallel with strains of low virulence

Table VI
Correlation between chronology of the bovine viral diarrhoea virus 2 species and genetic variation

Geno-type	Sub-genotype	Var[a]	Strain	Origin	Country	Year of isolation	Clinical presentation
BVDV-2a	1	2	BD-78	Sheep	United States	1978	Border disease
BVDV-2a	1	1	713-2	Cattle	United States	1982	Foetal abortion
BVDV-2a	1	3	Lees	Sheep	United Kingdom	1985	Border disease
BVDV-2a	1	3	167 237	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	3	168 149	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	3	173 157	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	3	175 375	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	6	CD87	Cattle	Canada	1987	Haemorrhagic syndrome
BVDV-2a	1	4	7937	Cattle	United States	1988	Persistent infection
BVDV-2a	2	–	SY-89	Cattle	Japan	1989	Persistent infection
BVDV-2a	2	–	OY89	Cattle	Japan	1989	Persistent infection, respiratory symptoms
BVDV-2a	1	5	890	Cattle	Canada	1990	Haemorrhagic syndrome
BVDV-2a	2	–	SW90	Cattle	Japan	1990	Mild symptoms
BVDV-2a	1	1	UVR420	Cattle	Belgium	1991	Calf intestine
BVDV-2a	1	4	BSE341	Cattle	Belgium	1991	Neurological symptoms
BVDV-2b	1	2	34b	Cattle	Argentina	1991	Foetus
BVDV-2b	2	–	Soldan	Cattle	Brazil	1991	Mucosal disease
BVDV-2a	2	–	TC Shinozaki	Cattle	Japan	1992	Persistent infection, respiratory symptoms
BVDV-2a	1	4	AZ Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	1	4	MAD Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	1	4	MN foetus	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	1	3	CPA	Contam[b]	Japan	1993	Not applicable
BVDV-2a	1	3	CPAE	Contam[b]	Japan	1993	Not applicable
BVDV-2a	1	3	EBTr	Contam[b]	Japan	1993	Not applicable
BVDV-2a	1	3	MMR-T	Contam[b]	Japan	1993	Not applicable
BVDV-2a	1	3	Rubella	Contam[b]	Japan	1993	Not applicable
BVDV-2a	1	4	23025	Cattle	United States	1993	Haemorrhagic syndrome
BVDV-2a	1	4	NY93	Cattle	United States	1993	Acute BVD
BVDV-2b	1	2	ncp7	Cattle	Argentina	1993	Foetus
BVDV-2a	1	3	Parvo	Contam[b]	Japan	1994	Not applicable
BVDV-2a	1	4	Q126	Cattle	Canada	1994	Haemorrhagic syndrome
BVDV-2a	1	4	15-103	Cattle	France	1994	Not available
BVDV-2a	1	4	WVD829	Cattle	Belgium	1994	Respiratory symptoms
BVDV-2a	1	6	4-5174	Cattle	France	1994	Not available
BVDV-2[c]	–	–	59386	Sheep	United Kingdom	1994	Border disease
BVDV-2[c]	–	–	SCP	Contam[b]	United Kingdom	1994	Not applicable
BVDV-2a	1	1	5521-95	Cattle	United States	1995	Foetal abortion

Table VI (contd)
Correlation between chronology of the bovine viral diarrhoea virus 2 species and genetic variation

Geno-type	Sub-genotype	Var[a]	Strain	Origin	Country	Year of isolation	Clinical presentation
BVDV-2a	1	3	BSE921	Cattle	Belgium	1995	Neurological symptoms
BVDV-2a	1	3	HE727	Contam[b]	Japan	1995	Not applicable
BVDV-2a	2	–	BS-95-II	Cattle	Italy	1995	Not available
BVDV-2d	–	–	354	Contam[b]	Argentina	1995	Not applicable
BVDV-2a	1	1	BSE1239	Cattle	Belgium	1996	Neurological symptoms
BVDV-2a	2	–	Giessen-1	Cattle	Germany	1996	Fatal mucosal disease
BVDV-2a	2	–	17011-96	Cattle	United States	1996	Foetal abortion
BVDV-2b	1	1	VS-63	Cattle	Brazil	1996	Healthy foetus
BVDV-2b	1	3	VS-123.4	Cattle	Brazil	1996	Healthy foetus
BVDV-2b	1	4	LV-96	Cattle	Brazil	1996	Chronic gastroenteric
BVDV-2a	1	1	97/730	Cattle	New Zealand	1997	Not available
BVDV-2a	1	4	11/Mi/97	Cattle	Italy	1997	Not available
BVDV-2b	1	4	VS-260	Cattle	Brazil	1997	Gastroenteric symptoms
BVDV-2a	1	4	17583-97	Cattle	United States	1997	Acute BVD
BVDV-2a	1	6	104/98	Cattle	Germany	1998	Mild symptoms
BVDV-2a	1	4	37Gr	Cattle	Austria	1998-2000	Acute diarrhoea
BVDV-2a	1	6	B52-2	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	1	6	Munich 1	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	1	6	Munich 2	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	B45-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	B50-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	B5-4	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	B77-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	Munich 3	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	AF112	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	1	4	IT-1732	Contam[b]	Italy	1999	Acute BVD
BVDV-2a	1	4	V-FLL	Contam[b]	Japan	1999	Not applicable
BVDV-2a	1	4	WG4622	Contam[b]	Netherlands	1999	Lethal acute BVD
BVDV-2a	2	–	MS-1	Cattle	Japan	1999	Persistent infection
BVDV-2a	1	6	i4083	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	1	6	i61380	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	1	6	i628	Cattle	Argentina	2001	Mild symptoms
BVDV-2c	–	–	i33283	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	1	2	C413	Sheep	USA	2002	Border disease
BVDV-2a	1	3	MP	Contam[b]	Belgium	2002	Not applicable
BVDV-2a	1	4	Kosice	Cattle	Slovakia	2002	Fatal course, malformations, haemorrhages

(a) variant
(b) contaminant
(c) not determined

Table VII
Observations on correlation between chronology of the bovine viral diarrhoea virus 2 species and genetic variation

Year	Observations
1978	First report of highly virulent BVDV-2a 1 2 in sheep in North America
1982	First report of low virulent BVDV-2a 1 1 in cattle in North America
1985	First report of highly virulent BVDV-2a 1 3 in sheep in UK
1987	First report of highly virulent BVDV-2a 1 6 in cattle in North America
1988	First report of highly virulent BVDV-2a 1 4 in cattle in North America The variant showed also rarely low virulence
1989	First report of low virulent BVDV-2a 2 in cattle in Japan
1990	First report of highly virulent BVDV-2a 1 5 in cattle in North America
1991	First report of low virulent BVDV-2a 1 1 in cattle in Continental Europe
1991	First report of low virulent BVDV-2a 1 4 in cattle in Continental Europe. This variant showed mainly high virulence in North America
1991	First report of low virulent BVDV-2b 1 2 in cattle in South America
1991	First report of MD BVDV-2b 2 in cattle in South America
1993	First report of contaminant BVDV-2a 1 3 in Japan
1994	First report of low virulent BVDV-2a 1 6 in cattle in Continental Europe
1995	First report of low virulent BVDV-2a 1 3 in cattle in Continental Europe
1995	First report of low virulent BVDV-2a 2 in cattle in Continental Europe
1995	First report of contaminant BVDV-2d in South America
1996	First report of low virulent BVDV-2b 1 1 in cattle in South America
1996	First report of low virulent BVDV-2b 1 3 in cattle in South America
1996	First report of low virulent BVDV-2b 1 4 in cattle in South America
1996	First report of low virulent BVDV-2a 2 in cattle in North America
1996	First report of MD BVDV-2a 2 in cattle in Continental Europe. This variant showed mainly low virulence in Europe and Japan
1997	First report of low virulent BVDV-2a 1 1 in cattle in New Zealand
1998-2000	First report of highly virulent BVDV-2a 1 4 in cattle in Continental Europe
1992-1999	First report of low virulent BVDV-2a 2 in cattle in Continental Europe
1999	First report of contaminant BVDV-2a 1 4 in Japan
2001	First report of low virulent BVDV-2a 1 6 in cattle in South America
2001	First report of low virulent BVDV-2c in cattle in South America

Kingdom, New Zealand and Japan. Field cattle strains from continental Europe, Japan and the United States, characterised 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 characterised by UG or UU, the strain from the United States showed UA. Genomic variation also distinguished between strains isolated from Brazil and Argentina. Within genotype 2b, Argentinean strains differed from their Brazilian counterparts at the level of the V2 locus in position 1 showing GU and AU, respectively. Argentinean strains belonging to genotypes 2b and 2d were characterised by V1/20 AA. Despite a similarity within genotype 2a, subgenotype 1, variant 6, Argentinean strains showed specific nucleotide changes compared to other strains from Europe and the United States and were clustered in the same variant. Argentinean cattle strains were similar to United States 2a 1 6 divergent V1/21, and similar to European divergent V1/1 showing GU instead of AU pairing. Similarly, strains affecting sheep showed divergence from those isolated from cattle (Fig. 4). They corresponded to variants 2 and 3 of subgenotype 1 within genotype 2a.

North America

1978 Highly virulent BVDV-2a 1 2 in sheep
1982 Low virulent BVDV-2a 1 1 in cattle
1987 Highly virulent BVDV-2a 1 6 in cattle
1988 Highly virulent BVDV-2a 1 4 in cattle
Variant also rarely showing low virulence
1990 Highly virulent BVDV-2a 1 5 in cattle
1996 Low virulent BVDV-2a 2 in cattle

United Kingdom

1985 Highly virulent BVDV-2a 1 3 in sheep

Continental Europe

1991 Low virulent BVDV-2a 1 1 in cattle
1991 Low virulent BVDV-2a 1 4 in cattle
Variant showing mainly high virulence
1994 Low virulent BVDV-2a 1 6 in cattle
1995 Low virulent BVDV-2a 1 3 in cattle
1995 Low virulent BVDV-2a 2 in cattle
1996 MD BVDV-2a 2 in cattle
Variant showing mainly low virulence
1998-2000 Highly virulent BVDV-2a 1 4 in cattle

Japan

1989 Low virulent BVDV-2a 2 in cattle
1993 Contaminant BVDV-2a 1 3
1999 Contaminant BVDV-2a 1 4

South America

1991 Argentina Low virulent BVDV-2b 1 2 in cattle
1991 Brazil MD BVDV-2b 2 in cattle
1995 Argentina Contaminant BVDV-2d
1996 Brazil Low virulent BVDV-2b 1 1 in cattle
1996 Brazil Low virulent BVDV-2b 1 3 in cattle
1996 Brazil Low virulent BVDV-2b 1 4 in cattle
2001 Argentina Low virulent BVDV-2a 1 6 in cattle
2001 Argentina Low virulent BVDV-2c in cattle

New Zealand

1997 Low virulent BVDV-2a 1 1 in cattle

Figure 2
Schematic presentation of observations on correlation between chronology of the BVDV-2 species and genetic variation

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 V31	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		CA							GC		UG		–
BVDV2 C413		.										AA	UG	UC		G	-	AU			CG		CA							GC		UG		–
BVDV2 713-2		.										AA	UG	CC		G	-	AU			UA				UG					GC			UU	–
Similar to 2a 1 1 New Zealand divergent V1/20 AU																																			
BVDV2 5521-95		.										AA	CG	CC		A	-	AU			UG									AU			UU	–
Similar to Argentine and European 2a 1 6 V1/21A																																			
BVDV2 CD87		.										AA	UG	CU		A	-	GU			UA	GU						GC		GU				–
Similar to 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 to European																																			
BVDV2 MAD Spl		GU										AA	UG	UU		G	-	AU			CG									GC		UG		–
BVDV2 MN Foetus		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	UU		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 V31	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 –
Brazilian cattle strains characterised by V1/12 GC V1/16 UA V1/17 GA or GG or AC V1/18 GG or GA V3 higher loop similar to 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			CU	U
BVDV2 VS-123.4		.					GC					UA	GA	GA	CG	GU	G	-	AU			UA		UG						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					UU	U
Argentinean cattle strains similar to Brazilian divergent V2/1 GU																																			
BVDV2 34b		.					GC					UA	GA	GA	CG		A	-	GU			UA		UG						AU			CG	CU	U
BVDV2 ncp7		.					GC					UA	GA	GA	CG		A	-	GU			UA		UG						AU			CG	CU	U
Argentinean strains characterised 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				CU	C
Argentinean cattle strains similar to United States 2a 1 6 divergent V1/21, similar to 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				–

Figure 3
Sequences in relation of geographic origin

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	UA	GC	CG	GU	AU	GU	CG		CG	CG	AU	UG	UC	–
Cattle strains characterised by V3/7 AC V3/8 UG or UU, similar to United States 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	–

United Kingdom

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	–
2a 1 3 similar to Belgian strain BSE921 divergent V1/17, 20																																				
BVDV2 167 237	.											AU	UA	CC		U	-	AU			UG									GC					–
BVDV2 168 149	.											AU	UA	CC		U	-	AU			UG									GC					–
BVDV2 173 157	.											AU	UA	CC		U	-	AU			UG									GC					–
BVDV2 175 375	.											AU	UA	CC		U	-	AU			UG									GC					–
BVDV2 Lees	.				GU							AU	UA	CC		U	-	AU			UG									GC					–

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 characterised 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 to United Kingdom divergent V2/5 AA																																				
BVDV2 MP	.											AU	UA	CC		U	-	AU			UG	AA								GC					–
Similar to United States 2a 1 6 divergent V1/21, similar to 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 to United Kingdom strains divergent V1/17, 20																																				
BVDV2 BSE921	.											AA	UA	AC		U	-	AU			UG									GC					–
2a 1 4 identical to 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 to United States 2a 1 1 divergent V1/20 AC																																				
BVDV2 97/730	.											AA	UG	CC	AC	A	-	AU			UG									AU					-

Figure 3 (contd)
Sequences in relation of geographic origin

The only strain affecting cattle showing a similarity with variant 1 3 was divergent in position 19 of V1. Among sequences from sheep, those from the United Kingdom were characterised by AU pairing in position 17, UA in position 18 and U in position 20 in the V1 locus. Those from the United States showed a characteristic CA bulge in position 6 of the V2 locus. Contamination of biological products was related to genotypes 2a and 2d.

Sheep

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 V31	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	–
BVDV2 167 237	.											AU	UA	CC		U	-	AU			UG								GC						–
BVDV2 168 149	.											AU	UA	CC		U	-	AU			UG								GC						–
BVDV2 173 157	.											AU	UA	CC		U	-	AU			UG								GC						–
BVDV2 175 375	.											AU	UA	CC		U	-	AU			UG								GC						–
BVDV2 Lees	.					GU						AU	UA	CC		U	-	AU			UG								GC						–
BVDV2 BD-78	.											AA	UG	UC		G	-	AU			CG		CA						GC		UG				–
BVDV2 C413	.											AA	UG	UC		G	-	AU			CG		CA						GC		UG				–
They correspond to variants 1 2 and 1 3																																			
The only strain affecting cattle showing relation being included in variant 1 3 is divergent in position 19 of V1																																			
BVDV2 BSE921	.											AA	UA	AC		U	-	AU			UG								GC						–

Cattle

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 V31	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	–
BVDV2 104/98	.											AA	UG	CU		G	-	GU			UA	GU					GC		GU						–
BVDV2 11/Mi/97	.											AA	CG	CC		G	-	AU			UG			UG					GC						–
BVDV2 15-103	.											AA	UG	UC		G	-	AU			CG								GC		UG				–
BVDV2 17011-96	.											AA	UG	CU		G	-	AC			UA								AU			AC	UA	UU	–
BVDV2 17583-97	.											AA	UG	UU		G	-	AU			CG								GC		UG				–
BVDV2 23025	.											AA	UG	UU		G	-	AU			CG								GC		UG				–
BVDV2 34b	.					GC					UA	GA	GA	CG		A	-	GU			UA		UG						AU				CG	CU	U
BVDV2 37Gr	.											AA	UG	UU		G	-	AU			CG								GC		UG				–
BVDV2 4-5174	.											AA	UG	CU		G	-	GU			UA	GU					GC		GU						–
BVDV2 5521-95	.											AA	CG	CC		A	-	AU			UG								AU					UU	–
BVDV2 713-2	.											AA	UG	CC		G	-	AU			UA			UG					GC					UU	–
BVDV2 7937	.											UA	UG	CU		G	-	AU			UG								GC						–
BVDV2 890	.											UA	UG	UC		G	-	AU			CG		CG			AC			GC						–
BVDV2 97/730	.											AA	UG	CC	AC	A	-	AU			UG								AU						–
BVDV2 AF112	.											AA	UG	CU		G	-	AC			UA								AU			AC	CA		–
BVDV2 AZ Spl	GU											AA	UG	CU	AC	G	-	AU			UG						GC		GU						–
BVDV2 B45-5	.											AA	UG	CU		G	-	AC			UA								AU			AC	CA		–
BVDV2 B50-5	.											AA	UG	CU		G	-	AC			UA								AU			AC	CA		–
BVDV2 B52-2	.											AA	UG	CU		G	-	GU			UA	GU					GC		GU						–
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 BSE1239	.											AA	UG	CU		G	-	AU			UG								AU						–
BVDV2 BSE341	.											AA	UG	CU		G	-	AU			UG						GC		GU						–
BVDV2 BSE921	.											AA	UA	AC		U	-	AU			UG								GC						–
BVDV2 CD87	.											AA	UG	CU		A	-	GU			UA	GU					GC		GU						–
BVDV2 Giessen-1	.											AA	UG	CU		G	-	AC			UA								AU			AC	CA		–
BVDV2 i33283	.						GU		CG			GA	GA	CA	AA	GG	U	GU			UA		UG						AU					CU	C
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						–
BVDV2 IT-1732	.											AA	UG	UC		G	-	AU			CG	GU							GC		UG				–
BVDV2 Kosice	.											AA	UG	UU		G	-	AU			CG								GC		UG				–
BVDV2 LV96	GA						GC				UA	GA	GG	CG		A	-	AU			UA		UG				AU		AU					CU	U
BVDV2 MAD Spl	GU											AA	UG	UU		G	-	AU			CG								GC		UG				–
BVDV2 MN FoetusGU												AA	UG	UC		G	-	AU			CG								GC		UG				–
BVDV2 MS-1	.											AU	UG	CU		G	-	AC			UA								AU			AC	UU		–
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 Munich 3	.											UA	CG	CU		G	-	AC			UA								AU			AC	CA		–
BVDV2 ncp7	.						GC				UA	GA	GA	CG		A	-	GU			UA		UG						AU				CG	CU	U
BVDV2 NY93	.											AA	UG	UC		G	-	AU			CG								GC		UG				–
BVDV2 OY89	.											AU	UG	CU		G	-	AC			UA								AU			AC			–
BVDV2 Q126	.											AA	UG	UC		G	-	AU			CG								GC		UG				–
BVDV2 Soldan	.						GC				UA	GG	GA	CA	AC	A	-	AU			UA		UG						AU	UA				CU	U
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			–
BVDV2 UVR420	.											AA	UG	CU		G	-	AU			UG						GC		AU						–
BVDV2 VS-123.4	.						GC				UA	GA	GA	CG	GU	G	-	AU			UA		UG						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					UU	U
BVDV2 WG4622	.											AA	UG	UC		G	-	AU			CG	GU							GC		UG				–
BVDV2 WVD829	.				GG							AA	UG	CU		G	-	AU			UG						GC		GU						–

Figure 4
Sequences of strains affecting sheep and cattle

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 to the haemorrhagic syndrome and apparently, as an exception, also variant 6 (Tables VIII and IX; Figs 5 and 6). Strains of high virulence that affect cattle were characterised by V1/19 UU or UC and V2/4 CG (stronger binding than UG detected in all strains of low virulence) except for two strains (AZ Spl and CD87). Similarly, ovine isolates from the United States showed the same characteristic. Ovine strains related to Border disease from the United Kingdom

Table VIII
Correlation between clinical features related with isolation of bovine virus diarrhoea virus 2 strains with genetic variation

Geno-type	Sub-genotype	Variant	Strain	Origin	Country	Year of isolation	Clinical presentation
Strains of high virulence							
BVDV-2a	1	2	BD-78	Sheep	United States	1978	Border disease
BVDV-2a	1	2	C413	Sheep	United States	2002	Border disease
BVDV-2a	1	3	Lees	Sheep	United Kingdom	1985	Border disease
BVDV-2a	1	3	167 237	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	3	168 149	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	3	173 157	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	3	175 375	Sheep	United Kingdom	1987	Border disease
BVDV-2a	1	4	AZ Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	1	4	MAD Spl	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	1	4	MN foetus	Cattle	United States	1960-1993	Haemorrhagic syndrome
BVDV-2a	1	4	23025	Cattle	United States	1993	Haemorrhagic syndrome
BVDV-2a	1	4	NY93	Cattle	United States	1993	Acute BDV
BVDV-2a	1	4	Q126	Cattle	Canada	1994	Haemorrhagic syndrome
BVDV-2a	1	4	17583-97	Cattle	United States	1997	Acute BVD
BVDV-2a	1	4	IT-1732	Contaminant	Italy	1999	Acute BVD
BVDV-2a	1	4	WG4622	Contaminant	Netherlands	1999	Lethal acute BVD
BVDV-2a	1	4	37Gr	Cattle	Austria	1998-2000	Acute diarrhoea
BVDV-2a	1	4	Kosice	Cattle	Slovakia	2002	Fatal course, malformations, haemorrhages
BVDV-2a	1	5	890	Cattle	Canada	1990	Haemorrhagic syndrome
BVDV-2a	1	6	CD87	Cattle	Canada	1987	Haemorrhagic syndrome
Strains responsible for mucosal disease in cattle							
BVDV-2a	2	-	Giessen-1	Cattle	Germany	1996	Fatal mucosal disease
BVDV-2b	2	-	Soldan	Cattle	Brazil	1991	Mucosal disease
Strains of low virulence							
BVDV-2a	1	1	713-2	Cattle	United States	1982	Foetal abortion
BVDV-2a	1	1	BSE1239	Cattle	Belgium	1996	Neurological symptoms
BVDV-2a	1	1	UVR420	Cattle	Belgium	1991	Calf intestine
BVDV-2a	1	1	5521-95	Cattle	United States	1995	Foetal abortion
BVDV-2a	1	1	97/730	Cattle	New Zealand	1997	Mild symptoms
BVDV-2a	1	3	BSE921	Cattle	Belgium	1995	Neurological symptoms
BVDV-2a	1	4	BSE341	Cattle	Belgium	1991	Neurological symptoms
BVDV-2a	1	4	7937	Cattle	United States	1988	Persistent infection

Table VIII (contd)
Correlation between clinical features related with isolation of bovine virus diarrhoea virus 2 strains with genetic variation

Geno-type	Sub-genotype	Variant	Strain	Origin	Country	Year of isolation	Clinical presentation
BVDV-2a	1	4	WVD829	Cattle	Belgium	1994	Respiratory symptoms
BVDV-2a	1	6	104/98	Cattle	Germany	1998	Mild symptoms
BVDV-2a	1	6	B52-2	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	1	6	Munich 1	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	1	6	Munich 2	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	1	6	i4083	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	1	6	i61380	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	1	6	i628	Cattle	Argentina	2001	Mild symptoms
BVDV-2a	2	–	17011-96	Cattle	United States	1996	Foetal abortion
BVDV-2a	2	–	SY-89	Cattle	Japan	1989	Persistent infection
BVDV-2a	2	–	OY89	Cattle	Japan	1989	Persistent infection, respiratory symptoms
BVDV-2a	2	–	TC Shinozaki	Cattle	Japan	1992	Persistent infection, respiratory symptoms
BVDV-2a	2		SW90	Cattle	Japan	1990	Mild symptoms
BVDV-2a	2	–	B45-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	B50-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	B5-4	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	B77-5	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	Munich 3	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	AF112	Cattle	Germany	1992-1999	Mild symptoms
BVDV-2a	2	–	MS-1	Cattle	Japan	1999	Persistent infection
BVDV-2b	1	1	VS-63	Cattle	Brazil	1996	Healthy foetus
BVDV-2b	1	2	34b	Cattle	Argentina	1991	Foetus
BVDV-2b	1	2	ncp7	Cattle	Argentina	1993	Foetus
BVDV-2b	1	3	VS-123.4	Cattle	Brazil	1996	Healthy foetus
BVDV-2b	1	4	LV-96	Cattle	Brazil	1996	Chronic gastroenteric
BVDV-2b	1	4	VS-260	Cattle	Brazil	1997	Gastroenteric symptoms
BVDV-2c	–	–	i33283	Cattle	Argentina	2001	Mild symptoms

showed characteristic UA pairing in V1/18 and U in V1/21 shared by only one cattle strain of low virulence. Sequences of cattle strains of low virulence showed bulges or CG pairing in V1/19 CC, CU, AC, CA and in V2/4 UG or UA.

The two nucleotides related to the degree of virulence in *Pestivirus* strains, described by Topliff and Kelling (47), referred to as N1 and N2, equivalent to nucleotide 219 and 278, respectively, according to the BVDV strain Osloss, were identifiable at the palindromic structures, variable loci V1 loop (for nucleotide 219) and V2 stem (for nucleotide 278) regions in the IRES. The nucleotide N1 corresponded to position 19 (left nucleotide) starting from the bottom of the palindrome, eight nucleotides in the V1 loop after the first cytosine of the *Pestivirus* characteristic CC bulge. Nucleotide N2 corresponded to position 4 (left nucleotide), one nucleotide after cytosine of the first *Pestivirus* characteristic C-G base-pair, characteristic of the genus *Pestivirus* in the V2 stem and five nucleotides before first guanine of the characteristic V2 loop sequence 5′-GGGGGU-3′. Taking into account the secondary palindromic structure at genus level, with the exception of the giraffe strain in which 58 nucleotides separated the

two virulence markers as in BVDV-2, in the other *Pestivirus* species, the distance varied due to the different distances between V1 and V2. In strains belonging to BVDV-1, the distance was 55 nucleotides (56 nucleotides in strain Deer UK 86) as in BDV and CSFV (54 nucleotides in the Brescia strain). Consequently, nucleotides that were apparently marking virulence were identified more clearly according to the position in the characteristic palindromic loci, avoiding confusion due to sequence variability.

BVDV-2 strains of high virulence were associated with two different nucleotide combinations, in the presence of uracil in position 219 and cytosine in position 278 (nine cattle isolates, including 890, two sheep isolates and two contaminants) and cytosine in position 219 and uracil in position 278 (two cattle isolates, including CD87, and five sheep isolates). In the BVDV-2 strains of low virulence, cytosine in position 219 and uracil in position 278 (37 cattle isolates, including two related to mucosal disease) and adenine in position 219 and uracil in position 278 (one cattle isolate) were the only combinations observed. No single strain was observed in the UC nucleotide combination which appeared to be related to high virulence alone.

Genotype BVDV-2a subgenotype 1 variant 2, 4 and 5 strains, including hyper-virulent strain 890 (variant 5), mainly presented uracil as N1 and cytosine as N2 (UC); all were related to high virulence (haemorrhagic syndrome or acute BVD). Within variant 4, six strains presented the CU combination, but only one was related to high virulence and the haemorrhagic syndrome. In variants 1 and 6, subgenotype 1, genotype BVDV-2a, only the combination of cytosine N1 and uracil N2 (CU) were evident, associated mainly with low virulence (abortion, respiratory or neurological symptoms or persistent infection), with the exception of strain CD87, associated with the haemorrhagic syndrome. Similarly, in genotype BVDV-2a subgenotype 2, CU was generally associated with mild clinical symptoms, apart from strain Giessen-1 that was associated with fatal mucosal disease. 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

Table IX
Summary of correlation between clinical features related with isolation of bovine virus diarrhoea virus 2 strains with genetic variation

Genotype	Subgenotype	Variant	Host	Clinical presentation	Number of observations
Strains of high virulence					
BVDV-2a	1	2	Sheep	Border disease	2
BVDV-2a	1	3	Sheep	Border disease	5
BVDV-2a	1	4	Cattle	Haemorrhagic syndrome	11
BVDV-2a	1	5	Cattle	Haemorrhagic syndrome	1
BVDV-2a	1	6	Cattle	Haemorrhagic syndrome	1
Strains responsible for mucosal disease in cattle					
BVDV-2a	2	-	Cattle	Fatal mucosal disease	1
BVDV-2b	2	-	Cattle	Mucosal disease	1
Strains of low virulence					
BVDV-2a	1	1	Cattle	Foetal 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

Position		V1	18	19	21	V2	1	4	5	6	V3	4	7	8	9	10
BVDV2									**AU**	**UA**			**AU**	**UG**	**UC**	**–**
Strains affecting cattle																
characterised by V1/19 UU or UC and V2/4 CG (stronger binding than UG detected in all low virulent strains)																
BVDV2	MAD Spl	.			UG	UU	G		AU	CG			GC			–
BVDV2	MN Foetus	.			UG	UC	G		AU	CG			GC			–
BVDV2	23025	.			UG	UU	G		AU	CG			GC			–
BVDV2	37Gr	.			UG	UU	G		AU	CG			GC			–
BVDV2	890	.			UG	UC	G		AU	CG		CG	GC			–
BVDV2	IT-1732	.			UG	UC	G		AU	CG	GU		GC			–
BVDV2	Kosice	.			UG	UU	G		AU	CG			GC			–
BVDV2	17583-97	.			UG	UU	G		AU	CG			GC			–
BVDV2	Q126	.			UG	UC	G		AU	CG			GC			–
BVDV2	NY93	.			UG	UC	G		AU	CG			GC			–
BVDV2	WG4622	.			UG	UC	G		AU	CG	GU		GC			–
Strains affecting sheep																
BVDV2	BD-78	.			UG	UC	G		AU	CG		CA	GC			–
BVDV2	C413	.			UG	UC	G		AU	CG		CA	GC			–
BVDV2	Lees	.			UA	CC	U		AU	UG			GC			–
BVDV2	167 237	.			UA	CC	U		AU	UG			GC			–
BVDV2	168 149	.			UA	CC	U		AU	UG			GC			–
BVDV2	173 157	.			UA	CC	U		AU	UG			GC			–
BVDV2	175 375	.			UA	CC	U		AU	UG			GC			–
2a Sg1, v4 V1/20 AC																
BVDV2	AZ Spl	.			UG	CU	G		AU	UG			GU			–
2a Sg1, v6 V1/21 A																
BVDV2	CD87	.			UG	CU	A		GU	UA	GU		GU			–
2a Sg2 MD (specific pathogenesis not related with high virulence)																
BVDV2	Giessen-1	.			UG	CU	G		AC	UA			AU	AC	CA	–
2b Sg2, MD (specific pathogenesis not related with high virulence)																
BVDV2	Soldan	.			GA	CA	A		AU	UA		UG	AU		CU	U

Figure 5
Sequences of highly virulent strains

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 a CU combination in relation with mild clinical course or contamination (genotype 2c), with the exception of subgenotype 2 of BVDV2-b that was related to mucosal disease.

In strains isolated from sheep (all animals with symptoms of Border disease), the presence of cytosine in position 219 and uracil in position 278 was the most frequent, in five out of seven strains, Lees, 167 237, 168 149, 173 157 and 175 375 (all strains originated from the United Kingdom). The presence of uracil in position 219 and cytosine in position 278 was observed in the two North American strains BD-78 and C413. In BVDV-2 strains, adventitious contaminants of biological products, in the presence of uracil in position 219 and cytosine in position 278, were observed in only two strains, the high virulence related strains WG4622 and IT-1732. All other tested strains, cell lines and biological products contaminants of unknown degrees of virulence showed cytosine in position 219 and uracil in position 278.

The virulence markers were highly variable in BVDV-1, BDV and CSFV strains. In BVDV-1, adenine as N1 and uracil as N2 (AU) revealed the most common nucleotide combination, with the exception of the BVDV-1a genotype with CU as the prevalent combination, in addition to AU, CC, UU, AC and GU combinations. The BVDV-1b genotype also presented GU, AC and CU combinations. GA and UC nucleotide pairing were also observed. BDV species showed UC as the most common combination and CC or UU; CSFV presented GC, CC or UC combinations; the giraffe strain showed an AU combination. With regard to high virulence related strains responsible for the haemorrhagic syndrome in European cattle (the BVDV-1d strains of the Culi series and strains Marloie and L256), these showed adenine as N1 and uracil as N2 (AU) and, in one case, uracil as N1 and N2 (UU).

Position			V1	18	19	21	V2	1	4	5	6	V3	4	7	8	9	10
BVDV2										**AU**	**UA**			**AU**		**UG**	**UC**
2a Sg1, v6 Similar sequences with CD87, divergence in position V1/21																	
BVDV2	i4083	.		CG	CU	G		GUUA		GU		GU					
BVDV2	i61380	.		CG	CU	G		GUUA		GU		GU					
BVDV2	i628	.		CG	CU	G		GUUA		GU		GU					
BVDV2	Munich 1	.		UG	CU	G		GUUA		GU		GU					
BVDV2	Munich 2	.		UG	CU	G		GUUA		GU		GU					
BVDV2	104/98	.		UG	CU	G		GUUA		GU		GU					
BVDV2	4-5174	.		UG	CU	G		GUUA		GU		GU					
BVDV2	B52-2	.		UG	CU	G		GUUA		GU		GU					
2a Sg2, identical with Giessen 1 MD																	
BVDV2	AF112	.		UG	CU	G		ACUA				AU		AC		CA	
BVDV2	B45-5	.		UG	CU	G		ACUA				AU		AC		CA	
BVDV2	B50-5	.		UG	CU	G		ACUA				AU		AC		CA	
BVDV2	B5-4	.		UG	CU	G		ACUA				AU		AC		CA	
BVDV2	Munich 3	.		CG	CU	G		ACUA				AU		AC		CA	
BVDV2	B77-5	.		UG	CU	G		ACUA				AU		AC		CA	
2a Sg1 4 Similar sequences with highly virulent strains 2a Sg1 4 divergence V1/19, V2/4																	
BVDV2	7937	.		UG	CU	G		AU UG				GC					
BVDV2	WVD829	.		UG	CU	G		AU UG				GU					
BVDV2	BSE341	.		UG	CU	G		AU UG				GU					
Other low virulent strains sequences																	
BVDV2	17011-96	.		UG	CU	G		ACUA				AU		AC	UA		UU
BVDV2	713-2	.		UG	CC	G		AU UA				GC					UU
BVDV2	BSE921	.		UA	AC	U		AU UG				GC					
BVDV2	5521-95	.		CG	CC	A		AU UG				AU					UU
BVDV2	97/730	.		UG	CC	A		AU UG				AU					
BVDV2	BSE1239	.		UG	CU	G		AU UG				AU					
BVDV2	UVR420	.		UG	CU	G		AU UG				AU					
BVDV2	VS-123.4	.		GA	CG	G		AU UA		UG		AU					CU U
BVDV2	LV96	.		GG	CG	A		AU UA		UG		AU					CU U
BVDV2	VS-260	.		GG	CG	A		AU UA		UG		AU					CU U
BVDV2	VS-63	.		GG	CG	A		AU UA		UG		AU					UU U
BVDV2	MS-1	.		UG	CU	G		ACUA				AU		AC	UU		
BVDV2	OY89	.		UG	CU	G		ACUA				AU		AC			
BVDV2	SW90	.		UG	CU	G		ACUA				AU		AC			
BVDV2	SY-89	.		UG	CU	G		ACUA				AU		AC			
BVDV2	TC Shinozaki	.		UG	CU	G		ACUA				AU		AC			
BVDV2	i33283	.		GA	CAGG			GUUA		UG		AU					CU C
BVDV2	ncp7	.		GA	CG	A		GUUA		UG		AU			CG	CU	U
BVDV2	34b	.		GA	CG	A		GUUA		UG		AU			CG	CU	U

Figure 6
Sequences of low virulent strains

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 enabled the identification of consensus motifs shared by all the *Pestivirus* species. BVDV-2 strains could be clearly differentiated genetically in terms of specific nucleotide substitutions by the BVDV-1 species. Four genotypes (BVDV-2a, BVDV-2b, BVDV-2c and BVDV-2d) were classified within BVDV-2 using the PNS method. South American isolates represented three 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 trade. However, between 1980 and 2002, limited bovine imports from Brazil 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). Importation of bubalines was also recorded (154 in 1998, 432 in 1999), but no sheep imports were made. In the same period, the number animals imported from Uruguay

increased (approximately 150 000 head for slaughter and 5 000 breeding animals). Despite the absence of epidemiological data, this could suggest a possible indirect source of virus spread in the region through a neighbouring country located between Brazil and Argentina.

Nomenclature for *Pestivirus* species is predominantly dependent on the species of host in 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. In the case of hog cholera virus (HCV), it was defined as being restricted to swine but natural infection in sheep was reported in Spain (27). Genetic analyses appeared necessary to provide the most appropriate approach for differential diagnosis to solve such cross-infections which may obscure the rationale for the definition of the *Pestivirus* species according to animal host. In our 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 were isolated from animals suffering from Border disease and presenting similar symptoms to those seen in cases affected by BDV, thus creating confusion in diagnosis. Among the BDV strains previously classified according to host animal species, the occurrence of other *Pestivirus* species has long been suspected (11, 14, 41). Technical terms 'true' BDV and 'atypical' BDV have been used to discriminate authentic BDV strains from false strains by Thiel and co-workers (1, 2, 3). 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, 46, 49). In a recent study using the PNS genotyping procedure, ovine isolates showed that the palindromic structure in the 5′-UTR was characteristic of BVDV-1a and BVDV-1b genovars and of BVDV-2 (19). The number of BVDV-2 ovine isolates reported to date is limited. In addition, it cannot be excluded that the five ovine strains reported by Vilcek *et al.* (49) were contaminants from foetal calf serum during cultivation of cells (50). Therefore, particular attention is required when performing cell isolations. The reverse transcriptase-polymerase chain reaction (RT-PCR) assay for the rapid recognition of virus in blood samples or original tissue homogenates with no cultivation on cells for Border disease clinical cases could be a useful approach.

Twelve BVDV-2 strains were detected as contaminants from cell cultures and biologicals. Four were reported from investigations performed in Europe, seven originated from Japan and one came from Argentina. The occurrence of BVDV-2 biological contamination of cell cultures, primary cell cultures and cell lines (including human cells) (1, 12, 21, 24) and vaccines for veterinary use (15, 21) is relevant. Previous reports have indicated BVDV contamination in vaccines for veterinary use (29, 31, 48, 54) but could not identify the BVDV species involved. Pestiviral RNA was detected in live virus vaccines for human use in Japan (21, 23). The most probable source of contamination is considered to be non-cytopathic BVDV infected bovine foetal serum commonly used as a medium supplement for the cell cultures (6, 8, 36). These findings indicate that contamination by animal pestiviruses may occur in biological products for veterinary and human use and that the problem appears to occur worldwide. Therefore, accurate and highly sensitive monitoring for adventitious *Pestivirus* should be recommended to manufacturers.

This concern and, in particular, the high virulence revealed by some BVDV-2 species strains causing the haemorrhagic syndrome in cattle is a characteristic that is also shared by BVDV-1 strains of the Culi series (Culi1, Culi4 and Culi6) (20, 30) and strains L256 and Marloie (32). In terms of nucleotide sequence, the definition of virulence activity among BVDV-2 species is still unclear. Similarly, BVDV-1 strains associated with the sporadic haemorrhagic syndrome could not be clustered in exclusively new subgroups (12). Using the two nucleotides related to the degree of virulence in *Pestivirus* strains, the palindromic structures, variable loci V1 loop

and V2 stem regions in the IRES in the 5′-UTR of the genomic RNA can be identified. Different nucleotide combinations could be identified. The most frequent combination was CU. UC and AU pairings were less frequent. The UC and CU nucleotide combinations associated with high virulence were in relation to genotype BVDV-2a. The CU nucleotide combination was shared with low virulence strains and was in relation to genotypes BVDV-2a, 2b and 2c. In cattle, uracil in position 219 and cytosine in position 278 were observed in 11 strains of severe clinical disease. The presence of cytosine in position 219 and uracil in position 278 was observed in 39 strains, with the exception of two strains that were mainly related to mild forms of clinical disease. The presence of cytosine at position 219 and uracil at position 278 were identified in two field isolates related to the haemorrhagic syndrome in North America. In one strain, adenine in position 219 and uracil in position 278 were observed. The observation made by Topliff and Kelling could be partially confirmed in our study. Out of the 13 highly virulent cattle strains tested, 84.61% revealed the suggested virulence marker UC. However, strains 890 and CD87 had to be considered of high virulence and low virulence, respectively, given that strain 890 presented uracil in position 219 and cytosine in position 278 and strain CD87 showed the two nucleotides in inverted locations, with cytosine at position 219 and uracil at position 278. Both strains were reported by Pellerin *et al.* (37) from outbreaks in Canadian cattle, thus confirming equal responsibility for the haemorrhagic syndrome. Similarly, strain AZ Spl that is responsible for the haemorrhagic syndrome in the United States, revealed both N1 C and N2 U, the alleged low virulence related nucleotide combination. The bovine strains Giessen-1, isolated during an outbreak of fatal mucosal disease in Germany, and Soldan isolated in Brazil in relation to clinical signs compatible with mucosal disease, were not associated with highly virulent strains in this study due to the specific pathogenesis of mucosal disease in immunotolerant animals (9).

In sheep, BVDV-2 was responsible for severe illness, irrespective of the presence of the UC or CU combination, thereby indicating the absence of a direct relationship with clinical manifestation of Border disease. In observations of positions 219 and 278, two North American isolates presented uracil and cytosine but those of five United Kingdom strains were inversed; it was suggested that these were caused by contamination (49).

In BVDV-2 strains contaminated by adventitious agents of biological products, the presence of uracil in position 219 and cytosine in position 278 was observed in only two strains and was related to high virulence. All other tested strains, cell lines and biological contaminants, with unknown degrees of virulence, showed cytosine in position 219 and uracil in position 278.

It seems appropriate to refer to the suggested nucleotide combination as a virulence marker. According to Topliff and Kelling, in the case of the iatrogenic accident that occurred in The Netherlands in cattle that had received a live virus vaccine contaminated with *Pestivirus* BVDV-2, genetic investigations performed by the State Institute indicated two specific nucleotide substitutions in the 5′-UTR as being characteristic of highly virulent BVDV-2 (15). However, this could raise the risk of underestimation for other nucleotide combinations that are potentially related to high virulence.

The high virulence characterised by the haemorrhagic syndrome in cattle is a relevant aspect in some BVDV-2 strains. Nevertheless, this characteristic was also observed in the Culi series strains (Culi1, Culi4 and Culi6) (20, 30) and strains L256 and Marloie (32) that belong to the BVDV-1 species, genotypes 1d and 1j, isolated from calves in Belgium. Experimental infection with BVDV-2 strains reproduced disease in naive calves, but this did not occur with BVDV-1 strains (20). This could suggest that induction of sporadic haemorrhagic syndrome by BVDV-1 strains requires the presence of a number of co-factors, whereas in the epidemic form of the haemorrhagic syndrome, BVDV-2 is the primary cause of the disease. However, the

degree of variation of virulence among strains is still unclear. For example, in Japan, BVDV-2 strains have been isolated since 1989, but a severe epidemic caused by BVDV 2 has never been recorded. In addition, in regard to BVDV-2, virulence might be the expression of an unknown factor of influence on the genetic structure of the virus. Therefore, further efforts are required to more fully understand the mechanism related to the generation of virulence in pestiviruses.

The observation of Pilipenko on the poliovirus 5′-UTR revealed several important aspects; a useful basis for investigations on virulence and the study of Topliff and Kelling revealed a similar relationship between the virulence characteristic and the IRES of BVDV-2. However, by increasing the number of strains evaluated, 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, still needs to be defined. Furthermore, the high variability in the other *Pestivirus* species is unclear, despite the genomic similarity to poliovirus, as for BVDV-2. In conclusion, uracil in position 219 and cytosine in position 278 appeared to be related to high virulence, but it was not always possible to determine virulence markers since high virulence was also associated with cytosine in positions 219 and 278 and cytosine in position 219 and uracil in position 278.

With 77-85 nucleotides, the palindromic loci represented a very limited portion of the virus genome. Within these short sequences, it was sufficient to obtain confirmed characterisation of the genus by evaluating only 18 nucleotides. Species were characterised through the evaluation of only 2 to 8 nucleotides. Similarly, the genotype was defined with only 2 to 6 nucleotides. These peculiar aspects resumed the high specificity of the PNS method and the reliability of the 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 economic importance of these viruses worldwide and the difficulties encountered in the control of the diseases, it is, therefore, important to understand the genetic aspects of the viruses and the history of the evolution of these viruses. These observations suggest that an evaluation of genomic secondary structure, by identifying markers of virus genomic characteristics in relation to the environment (geographic segregation as well as animal host) and clinical course, may be applied as a useful tool for epidemiological evaluation, thereby improving our understanding of the BVDV-2 species epizootiology, and possibly other species of the genus *Pestivirus* as well.

Acknowledgments

The authors express their thanks to F. Maillet of the *Clinique Saint François* in Ambilly, France, for valuable technical assistance and to L. Jones of the *Instituto de Virologia, Centro de Investigacion en Ciencias Veterinarias y Agronomicas* (CICVyA), *INTA-Castelar* in Castelar, Buenos Aires.

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