NEW MICROBIOLOGICA, **31**, 263-271, 2008

Genetic typing of bovine viral diarrhoea virus: evidence of an increasing number of variants in Italy

Sara Ciulli[1], Elena Galletti[1], Mara Battilani[1], Alessandra Scagliarini[1], Arcangelo Gentile[2], Luigi Morganti[1], Santino Prosperi[1]

[1]Department of Veterinary Public Health and Animal Pathology, Alma Mater Studiorum, University of Bologna, Italy; [2]Veterinary Clinical Department, Alma Mater Studiorum, University of Bologna, Italy

SUMMARY

Bovine Viral Diarrhoea Virus (BVDV) is responsible worldwide for severe economic losses on cattle farms. BVDV is an RNA virus with a high genome variability having practical consequences on epidemiology, diagnosis and disease control. Genetic monitoring was suggested as the first step in BVDV control. Thirty-seven Bovine Viral Diarrhoea Viruses were identified in persistently infected cattle, mucosal disease-affected animals and in bulk milk, and were characterised genetically. The 5'UTR region was amplified and sequenced, and a phylogenetic analysis was carried out comparing all the Italian sequences of BVDV available from the Genbank database. An unusual number of persistent infected animals was evidenced on more than one farm. Phylogenetic analysis attributed all our viruses to BVDV type I and distinguished four different subgroups inside this genotype. Analysis of old and new viruses revealed the circulation of viruses classified in subgroups BVDV Ia and Ij never reported in Italy.

KEY WORDS: Bovine viral diarrhoea virus, BVDV/MD, Persistent infection, Genetic typing, Phylogenetic analysis

Received October 30, 2007 *Accepted* December 13, 2007

INTRODUCTION

Bovine viral diarrhoea virus (BVDV) infection is present worldwide in cattle farms and the BVDV was found wherever it was searched for. Following BVDV infection, both respiratory and enteric symptoms can be observed on cattle farms, while on milk production farms bovine viral diarrhoea virus (BVDV) is mainly responsible for reproductive failure causing severe economic losses. The BVDV is a ssRNA+ virus of the genus *Pestivirus* in the family *Flaviviridae* (Fauquet *et al.,* 2005). Like most of the RNA viruses, it has a high evolution rate due to the infidelity of the RNA-dependent RNA polymerase which lacks proof-reading activity. As a consequence of its high mutation rate, the BVDV can present itself as variants with different pathogenicity and antigenicity, thus complicating the diagnosis and the control of the disease (Fulton *et al.,* 2003; Konig *et al.,* 2003; Bolin and Grooms, 2004). BVDV variability can also be used in molecular epidemiology as the multiple BVDV type I variants have a different geographical distribution (Vilcek *et al.*, 2001). Furthermore, immunotolerance induces a herd-specific BVDV strain allowing the identification of foreign strains (Paton *et al.*, 1995). The infection is maintained on the farm by persistent infected animals (PI) that are immunotolerant and genetic variants are generally limited by immune selection. PI prevalence is

Corresponding author
Sara Ciulli
Department of Veterinary Public Health
and Animal Pathology
Alma Mater Studiorum
University of Bologna,
Via Tolara di Sopra, 50
40064 Ozzano Emilia (BO), Italy
E-mail: sara.ciulli@unibo.it

normally equal or less than 2%, even if sporadic higher prevalence was evidenced in some USA regions (Houe, 2003). Genetic monitoring can guarantee the knowledge of pre-existent variants and can reveal the introduction of foreign viruses, both into the farm or into the national boundaries. Several countries have undertaken genetic monitoring to map BVD viruses present in their territories (Sakoda *et al.,* 1999; Tajima *et al.,* 2001; Couvreur *et al.,* 2002; Hurtado *et al.,* 2003; Vilcek *et al.,* 2003a; Vilcek *et al.,* 2003b; Mishra *et al.,* 2004; Parks *et al.,* 2004; Toplak *et al.,* 2004; Mahony *et al.,* 2005; Stalder *et al.,* 2005; Tajima and Dubovi 2005). The present study has the aim to further analyse Italian BVDV strains, continuing genetic monitoring and to better characterise the strains isolated to date.

MATERIALS AND METHODS

Animals and viruses

Thirty-seven BVD viruses were collected in Italy between 2000 and 2005 and genetically analysed. Thirty-five viruses were detected from blood or organ samples of PI calves or mucosal disease (MD) affected animals and 2 viruses were detected from bulk milk. PI animals were detected by a two-step analysis: first a serological screening was conducted and then seronegative animals were tested twice a month apart using an RT-PCR assay. On some infected farms, newborn calves were monitored for persistent infection for one year. MD affected animals were PI animals which showed typical erosive symptoms in the gastrointestinal apparatus and in the feet. Numbers of animals tested in each farms are listed in table 1. Samples were collected from 10 farms with reproductive problems located in North Italy, 6 in the province of Bologna, 1 in the province of Modena, 2 in the province of Vicenza and 1 in the province of Padua. Details of the viruses are given in Table 2.

The viruses were characterised directly from the RNA extracted from the samples without cell culture adaptation to avoid mutations due to in vitro passages.

RNA extraction, cDNA synthesis and amplification

Viral RNA was extracted from 140 μL of plasma using the QIAamp Viral RNA Mini Kit (QIAgen) according to the instruction manual and was conserved at -80°C until use. RNA was extracted from the somatic cells of 500 mL of bulk milk and from 50 mg of tissue using TRI reagent (Sigma). Cells or tissues were briefly homogenized with quartz powder and 500 μL of TRI reagent, centrifuged at

TABLE 1 - *The number of animals tested on each farm. Animals from farms M to P were already serologically screened and they were subjected directly to RT-PCR for BVDV.*

Farm	*N° of animals serologically screened*	*Seropositive animals*	*Seronegative animals*	*Seronegative animals positive to BVDV by RT-PCR*	*Animals tested directly by RT-PCR*	*Newborn animals positive to BVDV by RT-PCR*	*Total number of viruses positive animals*
A/00	124	120	4	3	/	/	3
B/01	50	41	9	7	6	1	8
C/01	48	41	7	1	/	/	1
G/02	60	8	52	0	29	3	3
M/03	/	/	/	/	4	4	4
N/03	/	/	/	/	2	2	2
O/05	/	/	/	/	13	13	13
P/05	/	/	/	/	1	1	1

TABLE 2 - *Details of the 37 BVD viruses analysed in the study; names refer to sample/herd/year.*

Name	*Sample*	*Symptoms*	*Province*	*Groups*
1/A/00	Spleen	PI from herd with reproductive failure	BO (Emilia R.)	Ib
2/A/00	Spleen	PI from herd with reproductive failure	BO (Emilia R.)	Ib
3/A/00	Spleen	PI from herd with reproductive failure	BO (Emilia R.)	Ib
1/B/01	Blood	PI from herd with reproductive failure and 1 MD animal	MO (Emilia R.)	Id
2/B/01	Blood	PI from herd with reproductive failure and 1 MD animal	MO (Emilia R.)	Id
3/B/01	Blood	PI from herd with reproductive failure and 1 MD animal	MO (Emilia R.)	Id
4/B/01	Blood	PI from herd with reproductive failure and 1 MD animal	MO (Emilia R.)	Id
5/B/01	Blood	MD	MO (Emilia R.)	Id
6/B/01	Blood	PI from herd with reproductive failure and 1 MD animal	MO (Emilia R.)	Id
7/B/01	Blood	PI from herd with reproductive failure and 1 MD animal	MO (Emilia R.)	Id
8/B/01	Blood	PI from herd with reproductive failure and 1 MD animal	MO (Emilia R.)	Id
1/C/01	Blood	PI from herd with reproductive failure	BO (Emilia R.)	Id
1/G/02	Blood	PI from herd with reproductive failure	BO (Emilia R.)	Ib
2/G/02	Blood	PI from herd with reproductive failure	BO (Emilia R.)	Ib
3/G/02	Blood	PI from herd with reproductive failure	BO (Emilia R.)	Ib
1/I/02	Bulk Milk	Pool from herd with reproductive failure	BO (Emilia R.)	Id
1/L/02	Bulk Milk	Pool from herd with reproductive failure	BO (Emilia R.)	If
1/M/03	Blood	PI from herd with reproductive failure	VI (Veneto)	Ib
2/M/03	Blood	PI from herd with reproductive failure	VI (Veneto)	Ib
3/M/03	Blood	PI from herd with reproductive failure	VI (Veneto)	Ib
4/M/03	Blood	PI from herd with reproductive failure	VI (Veneto)	Ib
1/N/03	Blood	PI from herd with reproductive failure	VI (Veneto)	Ia
2/N/03	Blood	PI from herd with reproductive failure	VI (Veneto)	Ia
1/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and MD	PD (Veneto)	Ia
2/O/05	Blood	PI from herd with reproductive failure, hemorrhagic enteritis and MD	PD (Veneto)	Ia
3/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and MD	PD (Veneto)	Ia
4/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and MD	PD (Veneto)	Ia
5/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and MD	PD (Veneto)	Ia
6/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and MD	PD (Veneto)	Ia
7/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and MD	PD (Veneto)	Ia
8/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and MD	PD (Veneto)	Ia
9/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and pneumonia	PD (Veneto)	Ia
10/O/05	Pleuric essudate	PI from herd with reproductive failure, respiratory symptoms and pneumonia	PD (Veneto)	Ia
11/O/05	Blood	PI from herd with reproductive failure, respiratory symptoms and pneumonia	PD (Veneto)	Ia
12/O/05	Blood	PI animal	PD (Veneto)	Ia
13/O/05	Blood	PI from herd with reproductive failure and respiratory symptoms and pneumonia	PD (Veneto)	Ia
1/P/05	Blood	MD with hemorrhagic enteritis	BO (Emilia R.)	Id

12,000 *x g* for 10 min at 4°C to remove insoluble debris. The supernatant was then added to 0.2 mL of chloroform and centrifuged at 12,000 *x g* for 15 min at 4°C. The aqueous phase containing the RNA was precipitated with isopropanol at room temperature and centrifuged as above. The pellet was washed in 1 mL of 75% ethanol, dried and suspended in 50 µL of Rnase-free water. The DNA was synthesized with Multiscribe Reverse Transcriptase (Applied Biosystem) with the addition of 1.25 µM random hexamers. A 288 bp fragment of the 5' UTR region was amplified with primers 324-326 as previously described (Vilcek *et al.,* 1994).

Sequencing and phylogenetic analysis

5'UTR amplification products were purified using the High Pure PCR Product Purification Kit (Roche) and then sequenced in both directions with the ABI 377 automatic sequencer (Applied Biosystem). Obtained sequences were aligned and compared with sequences available on GenBank using Clustal W (Thompson *et al.*, 1994) of the Lasergene Biocomputing software (DNASTAR Inc. Madison). The alignment was manually edited with the BIOEDIT program ver. 5.0.9 (Hall, 1999) and phylogenetic analysis was carried out using the Phylip program ver 3.6 (Felsenstein, 2001). The pairwise genetic distance was calculated using the Felsestein 84 (F84) method and a phylogenetic tree was constructed using the Neighbour-joining method. Bootstrap analysis was carried out on 1000 replicates (Felsenstein, 1985).

Genbank accession numbers

Nucleotide sequences reported in the Genbank database had the following accession numbers: *AY451338, AY451339*.

RESULTS

Animals

From one to thirteen PI animals were found on infected farms with an average percentage of 3.9 PI animals per farm. In 4 (B, M, N and O) out of 8 farms we found a high percentage of PI animals (Table 3). On all the farms except 2 (G and O) the PI animals were detected at the same time and generally they had the same age. On farm G sample 3/G/02 was collected 1 year apart from the others, and on farm O samples 12/O/05 and 13/O/05 were collected two months after the elimination of all the other infected animals from the farm. Furthermore, on farm O the PI group was composed of animals with an age between 1 and 8 months. MD affected animals were present in farm B, O and P. On farm O all the animals except 1 died naturally with typical MD form or due to secondary infections (pneumonia, septicaemia).

TABLE 3 - *Prevalence of PI animals on analysed farms.*

Farm	*Animal number/herd*	*Time of sampling*	*PI animals*	*Prevalence %*
A/00	160	2000	3	1.9
B/01	120	2001	8	6.6
C/01	160	2001	1	0.6
G/02	110	2002 September 2003 March	2 1	1.8 0.9
M/03	50	2003	4	8
N/03	55	2003	2	3.6
O/05	100	2005 February 2005 May	11 2	11 2
P/05	60	2005	1	1.6

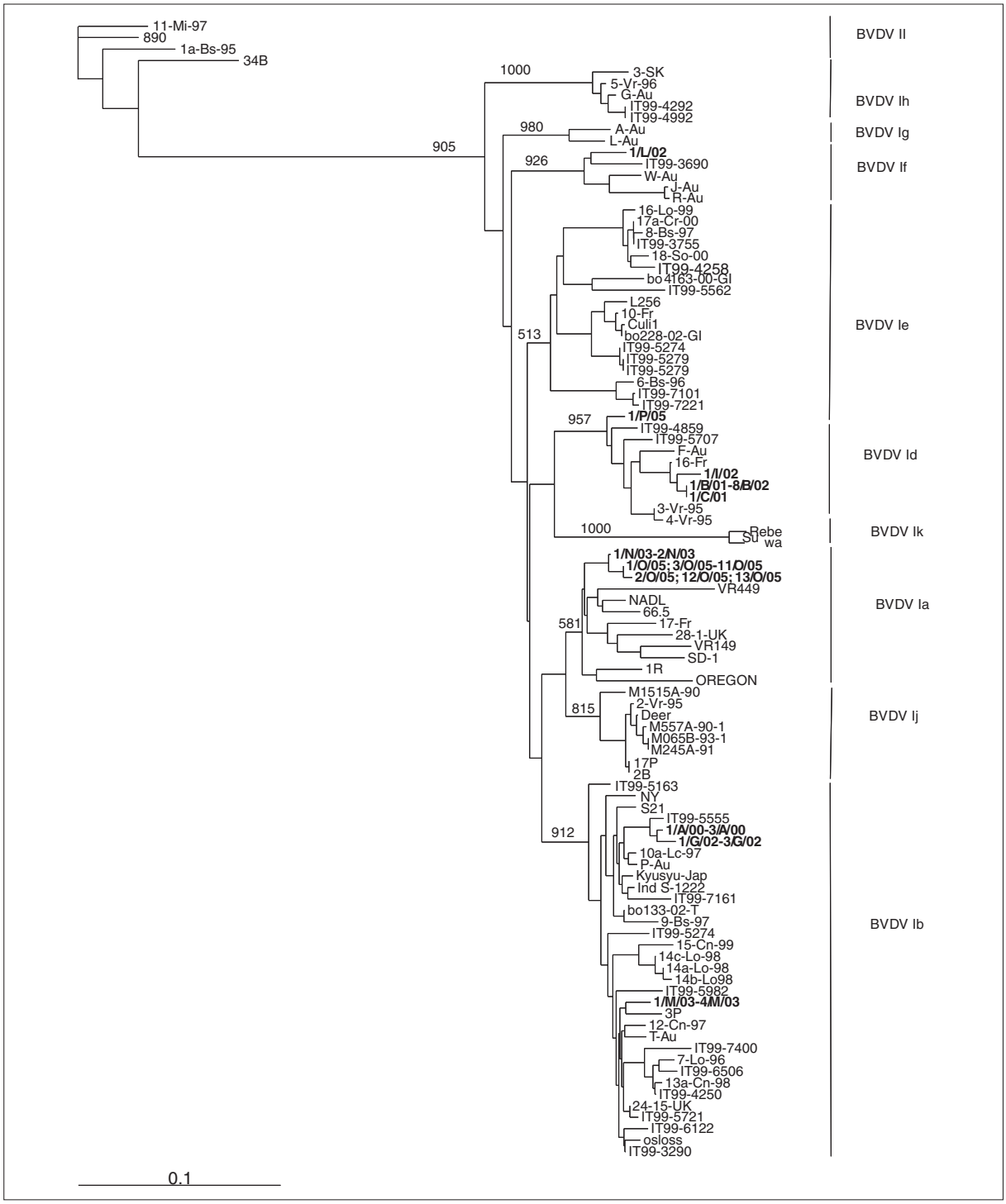

FIGURE 1 - *Phylogenetic analysis of BVDV strains from Italy and other geographical regions (strains sequenced in this work are labeled in bold print). Sequences for other strains were acquired from the GenBank (Acces. Nos. NADL - M31182; Oregon - AF091605, SD - 1 - M96751, Osloss - M96687; NY - L32879, 890 - L32886; Rebe - AF299317; Suwa - AF117699; Deer - AB040132); the remaining sequences have previously been published (Baule et al., 1997; Sakoda et al., 1999; Luzzago et al., 2001; Vilcek et al., 2001; Couvreur et al., 2002; Falcone et al., 2003; Hurtado et al., 2003; Jones et al., Mishra et al., 2004; Mahony et al., 2005). Main bootstrap values are shown.*

Genetic amplification and characterization

A specific amplification product of 288 bp was obtained for all viruses and the corresponding sequences were analysed.

Comparing our sequences with reference strains, all the studied viruses belonged to BVDV I genotype and BVDV type II was not found among the Italian analysed strains.

On 7 out of 10 farms more than one PI animal was present: 100% identity was disclosed among viruses from animals of the same herd on 6 farms out of 7. On farm O, 3 out of 13 strains showed 1 nucleotide substitution. On this farm, the viruses were collected from animals which were infected over a long period of time (11-12 months). so the virus could have changed during serial infections from one animal to another. Sequence similarity of the strains from different farms ranged between 89.4 and 100%.

Comparing our sequences with Italian, European and worldwide strains (Figure 1), we attributed them to different subgroups of the BVDV I genotype and we could calculate the prevalence of different subgroups in Italy. Ten viruses from 3 farms were clustered in Group I-b together with reference strains NY-1, Osloss and most of the Italian strains (43 out of 101) previously isolated (Luzzago *et al.,* 2001; Falcone *et al.,* 2003); 11 viruses from 4 farms were clustered in the Id group with the 3/Vr/95 and 4/Vr/95 strains detected by Luzzago *et al.* (2001) in Italy and not yet classified. Fifteen viruses from another 2 farms located in the Veneto region clustered with reference strains SD-1 and NADL and were BVDV Ia (Figure 2). Finally, one of the two viruses detected in bulk milk was attributed to group If and it clustered with an Austrian and another Italian strain.

No Ie subgroup viruses were detected, even if it was one of the most prevalent (26.3%) in a previous study (Falcone *et al.*, 2003). Most of the farms analysed (40%) were infected with subgroups Id; 30% of the farms were infected with Ib, 20% were infected with Ia and 10% of the outbreaks were due to the If strain; therefore, Id and the Ib strains are the most prevalent subgroups in the area of our study. The percentages of identity inside each subgroup were >95.5% (Ib), >96.2% (Id) and >99% (Ia). The strain If shared an identity of 95.8% with the Italian strain IT99-3690 which had previously been isolated (Falcone *et al.*, 2003). Most of the other Italian strains were confirmed to be Ib, Ie, Id, Ih and If as previously classified, and some unclassified strains could be clustered in already known subgroups. Strain 2/Vr/95 clustered with the virus "Deer" and with African (Baule *et al.,* 1997) and Argentinean (Jones *et al.,* 2001) strains. In a previous study these strains were attributed to subgroup Ij (Vilcek *et al.,* 2004), in spite of a low bootstrap value.

In our phylogenetic analysis this cluster was supported by a high bootstrap value (81.3%) confirming that these strains form a distinct lineage. Strains 3/Vr/95 and 4/Vr/95 clustered with Id strains, strain 5/Vr/95 with Ih and strains 6/Bs/96, 8/Bs/97; 16/Lo/99, 17a/Cr/00, 17b/Cr/00, 18/So/00 clustered with BVDV Ie. When all Italian strains were considered, final percentages of the different groups (Luzzago *et al.,* 2001; Vilcek *et al.,* 2001; Falcone *et al.,* 2003) were 43.56% for Ib, 18.81% for Id, 15.84% for Ie, 14.85% for Ia, 1.98% for If, 0.99% for Ih, and 0.99% for Ij; 1.98% of viruses detected were genotype II. No viruses of groups Ic, Ig, Ii, and Ik have been detected in Italy to date.

FIGURE 2 - *Geographic distribution of BVDV strains analyzed in this study.*

DISCUSSION

The presence of BVDV infection in the regions analysed in this investigation is well-known and of notable entity and this study confirms the presence of BVDV on farms with reproductive problems. Despite literature reports that PI prevalence is generally equal or less than 2% (Houe, 2003), we found a higher number of PI animals on 4 out of 8 analysed farms. In particular, we found 8 PI animals of the same age on farm G and 13 one to 8 months old PI animals on farm O. In our opinion this high number of PI animals of the same age on the same farm could be due to the entry of the infection in the farm with pregnancy synchronisation, whereas many PI with different ages could be due to incorrect management of sanitary control, particularly the lack of a correct separation of newborn animals and pregnant cows.

Many authors (Vilcek *et al.,* 1999; Nagai *et al.,* 2004) have recognized that phylogenetic analysis is useful in studying the evolutionary and epidemiological history of a virus and in tracing the origin of new outbreaks. The effects of BVDV variability on diagnosis and control are well-known (Fulton *et al.*, 2003; Konig *et al.,* 2003; Bolin and Grooms, 2004) and ongoing studies are attempting to increase knowledge on genotype and subgroup distribution worldwide (Parks *et al.,* 2004; Tajima *et al.,* 2005).

This study genetically analysed 37 viruses collected from 10 farms in 2 regions of Northern Italy. High homology was observed among viruses detected in each farm. The selection of one strain per farm had previously been evidenced (Paton *et al.,* 1995) and it is typical of BVDV infection due to immunotolerance. Viral strains are, in fact, selected on the basis of the strain present in immunotolerant animals while variants are disadvantaged due to the ability of immunotolerant and immunocompetent animals to react and eliminate them after a transient infection.

Furthermore, genetic analysis showed that no BVDV II has recently been detected, despite the fact that it was found twice in different areas of North Italy (Luzzago *et al.,* 2001).

Two previous studies detected and characterised Italian BVDV strains, but they never found the Ia group (Luzzago *et al.,* 2001; Falcone *et al.,* 2003). Furthermore, some viruses remained unclassified. Our phylogenetic analysis permitted us to attribute all the Italian viruses detected to specific subgroups by demonstrating not only BVDV Ia, but also Ij which had not been reported until now. In our opinion, only the collection of a remarkable number of strains can permit a consistent classification.

Our study failed to find any correlation with MD outbreaks and other symptoms or non-symptomatic forms from different subgroup viruses despite the fact that some correlation had been observed in previous studies (Evermann and Ridpath, 2002; Fulton *et al.,* 2002). BVDV Ia outbreaks observed on farm G showed a more severe course and all the PI animals except one died; most of them (8 animals) showed a typical MD form with multiple oral lesions and hemorrhagic enteritis and the other 4 died from pneumonia and septicaemia due to secondary infection. This evolution however could be favoured by bad management and, above all, by the fact that after their detection PI animals were stabled separately. After the onset of the MD the symptoms spread quickly from one animal to another and death occurred in 3 to 5 days.

As regards the geographic distribution, all the BVDV Ia strains and a BVDV Ib were detected in Veneto. We also noted that strains Ij, Id and Ih previously detected by Luzzago *et al.* also came from this regions (2001) and showed a general higher heterogeneity compared to Emilia-Romagna and Lombardia strains.

Lastly, BVDV Ib is currently prevalent in Italy, but in our study, the total percentage of viruses in subgroups other than Ib (72.9%) were prevalent over the Ib (27.02%) viruses, and we found an emerging circulation of foreign groups, such as Ia, and Ij, which had never been detected in Italy to date and were widespread mainly in non-European countries. The lack of a mandatory control program for BVDV could increase not only BVD outbreaks, but also the circulation of new variants in the near future. To avoid this it is indispensable to adopt an identification and culling protocol for PI animals.

CONCLUSION

Genetic characterisation is the basic process to have a true vision of virus evolution, especially

for highly variable RNA viruses like BVDV. Nowadays there is an available and growing knowledge of BVDV distribution in Europe, the USA and other countries. Subsequent studies on the Italian situation showed an increasing number of variants nationwide. High heterogeneity could be concentrated mainly in some regions. The importance of this outcome needs to be considered to implement an appropriate control programme. First of all the ongoing evidence of viruses classified in new subgroups emphasizes the importance of continued molecular surveillance as the basis of BVDV control.

ACKNOWLEDGEMENTS

The authors thank Dr. Cazzoli M., Dr. Zocca A. and Dr. Zin G. for providing BVDV affected animals. We also thank Gerald Goldsmith for linguistic improvement of the manuscript.

REFERENCES

BAULE C., VAN VUUREN M., LOWINGS J.P., BELAK S. (1997). Genetic heterogeneity of bovine viral diarrhea viruses isolated in Southern Africa. *Virus Res.* **52**, 205-220.

BOLIN S.R., GROOMS D.L. (2004). Origination and consequences of bovine viral diarrhea virus diversity. *Vet. Clin. North Am. Food Anim. Pract.* **20**, 51-68.

COUVREUR B., LETELLIER C., COLLARD A., QUENON P., DEHAN, P., HAMERS C., PASTORET P.P., KERKHOFS P. (2002). Genetic and antigenic variability in bovine viral diarrhea virus (BVDV) isolates from Belgium. *Virus Res.* **85**, 17-28.

EVERMANN J.F., RIDPATH J.F. (2002). Clinical and epidemiologic observations of bovine viral diarrhea virus in the northwestern United States. *Vet. Microbiol.* **89**, 129-139.

FALCONE E., CORDIOLI P., TARANTINO M., MUSCILLO M., LA ROSA G., TOLLIS M. (2003). Genetic heterogeneity of bovine viral diarrhoea virus in Italy. *Vet. Res. Commun.* **27**, 485-494.

FAUQUET C.M., MAYO M.A., MANILOFF J., DESSELBERGER U., BALL L.A. (2005). Virus taxonomy, Classification and Nomenclature of Viruses. Eighth ICTV Report, Academic Press, San Diego, California, USA.

FELSENSTEIN J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791.

FELSENSTEIN J. (2001). Phylogenetic inference package 3.6 (alpha3). Distributed by the author, Department of Genetics, University of Washington, Seattle.

FULTON R.W., RIDPATH J.F., SALIKIM J.T., BRIGGS R.E., CONFER A.W., BURGE L.J., PURDY C.W., LOAN R.W., DUFF G.C., PAYTON M.E. (2002). Bovine viral diarrhea virus (BVDV) 1b: predominant BVDV subtype in calves with respiratory disease. *The Canadian Journal of Vet. Res.* **66**, 181-190.

FULTON R.W., RIDPATH J.F., CONFER A.W., SALIKI J.T., BURGE L.J., PAYTON M.E. (2003). Bovine viral diarrhea virus antigenic diversity: impact on disease and vaccination programmes. *Biologicals* **31**, 89-95.

HALL T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* **41**, 95-98.

HOUE H. (2003). Economic impact of BVDV infection in dairies. *Biologicals* **31**, 137-143.

HURTADO A., GARCIA-PEREZ A.L., ADURIZ, G., JUSTE R.A. (2003). Genetic diversity of ruminant pestiviruses in Spain. *Virus Res.* **92**, 67-73.

JONES L.R., ZANDOMENI R., WEBER E.L. (2001b). Genetic typing of bovine viral diarrhea virus isolates from Argentina. *Vet. Microbiol.* **81**, 367-375.

KONIG M., CEDILLO, ROSALES S., BECHER P., THIEL H.J. (2003). Heterogeneity of ruminant pestiviruses: academic interest or important basis for the development of vaccines and diagnostics? *Berl. Munch. Tierarztl. Wochenschr.* **116**, 216-221.

LUZZAGO C., BANDI C., BRONZO V., RUFFO G., ZECCONI A. (2001). Distribution pattern of bovine viral diarrhoea virus strains in intensive cattle herds in Italy. *Vet. Microbiol.* **83**, 265-274.

MAHONY T.J., MCCARTHY F.M., GRAVEL J.L., CORNEY B., YOUNG P.L., VILCEK S. (2005). Genetic analysis of bovine viral diarrhoea viruses from Australia. *Vet. Microbiol.* **106**, 1-6.

MISHRA N., PATTNAIK B., VILCEK S., PATIL S.S., JAIN P., SWAMY N., BHATIA S., PRADHAN H.K. (2004). Genetic typing of bovine viral diarrhoea virus isolates from India. *Vet. Microbiol.* **104**, 207-212.

NAGAI M., HAYASHI M., SUGITA S., SAKODA Y., MORI M., MURATAMI T., OZAWA T., YAMADA N., AKASHI H. (2004). Phylogenetic analysis of bovine viral diarrhea viruses using five different genetic regions. *Virus Res.* **99**, 103-113.

PARKS J.S., MOON H.J., LEE B.C., HWANG W.S., YOOM H.S., KIMM D.Y., PARK B.K. (2004). Comparative analysis of the 5'-untraslated region of bovine viral diarrhea virus isolated in Korea. *Res. Vet. Science.* **76**, 157-163.

PATON D.J., CARLSSON U., LOWINGS J.P., SANDS J.J., VILCEK S., ALENIUS S., (1995). Identification of herd-specific bovine viral diarrhea virus isolates from infected cattle and sheep. *Vet. Microbiol.* **43**, 283-294.

SAKODA Y., OZAWA S., DAMRONGWATANAPOKIN S., SATO M., ISHIKAWA K., FUKUSHO A. (1999). Genetic heterogeneity of porcine and ruminant pestiviruses mainly isolated in Japan. *Vet. Microbiol.* **65**, 75-86.

STALDER H.P., MEIER P., PFAFFEN G., WAGECK-CANAL C.,

Rufenacht J., Schaller P., Bachofen C., Marti S., Vogt H.R., Peterhans E. (2005). Genetic heterogeneity of pestiviruses of ruminants in Switzerland. *Prev. Vet. Med.* **72**, 37-41.

Tajima M., Frey H.R., Yamato O., Maede Y., Moennig V., Scholz H., Greiser-Wilke I. (2001). Prevalence of genotypes 1 and 2 of bovine viral diarrhea virus in Lower Saxony, Germany. *Virus Res.* **76**, 31-42.

Tajima M., Dubovi E.J. (2005). Genetic and clinical analyses of bovine viral diarrhea virus isolates from dairy operations in the United States of America. *J. Vet. Diagn. Invest.* **17**, 10-15.

Thompson J.D., Higgins D.G., Gibson T.J. (1994). CLUSTAL W: improving the sensivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Toplak I., Sandvik T., Barlic-Maganja D., Grom J., Paton D.J. (2004). Genetic typing of bovine viral diarrhoea virus: most Slovenian isolates are of genotypes 1d and 1f. *Vet. Microbiol.* **99**, 175-185.

Vilcek S., Herring A.J., Herring J.A., Nettleton P.F, Lowings J.P, Paton D.J. (1994). Pestivirus isolated from pigs, cattle and sheep can be allocated into three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch. Virol.* **136**, 309-323.

Vilcek S., Drew T.W., McGoldrick A., Paton D.J. (1999). Genetic typing of bovine Pestivirus from England and Wales. *Vet. Microbiol.* **69**, 227-237.

Vilcek S., Paton D.J., Durkovic B., Strojny L., Ibata G., Moussa A., Loitsch A., Rossmanith W., Vega S., Scicluna M.T., Palfi V. (2001). Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. *Arch. Virol.* **146**, 99-115.

Vilcek S., Greiser-Wilke I., Durkovic B., Obritzhauser W., Deutz A., Kofer J. (2003a). Genetic diversity of recent bovine viral diarrhoea viruses from the southeast of Austria (Styria). *Vet. Microbiol.* **91**, 285-291.

Vilcek S., Mojzisova J., Bajova V., Paulik S., Strojny, L., Durkovic B., Hipikova V. (2003b). A survey for BVDV antibodies in cattle farms in Slovakia and genetic typing of BVDV isolates from imported animals. *Acta Vet. Hung.* **51**, 229-236.

Vilcek S., Durkovic B., Kolesarova M., Greiser-Wilke I., Paton D. (2004). Genetic diversity of international bovine viral diarrhoea virus (BVDV) isolates: identification of a new BVDV-1 genetic group. *Vet. Res.* **35**, 609-615.