Journal of General Virology (2012), **93**, 1976–1983 **DOI** 10.1099/vir.0.044552-0

Hobi-like pestivirus: both biotypes isolated from a diseased animal

Nicola Decaro,[1] Viviana Mari,[1] Pierfrancesco Pinto,[1] Maria Stella Lucente,[1] Rossana Sciarretta,[1] Francesco Cirone,[1] Maria Loredana Colaianni,[1] Gabriella Elia,[1] Heinz-Jürgen Thiel[2] and Canio Buonavoglia[1]

[1]Department of Veterinary Public Health, University of Bari, Valenzano (Bari), Italy

[2]Institut für Virologie, Justus Liebig Universität Giessen, Giessen, Germany

Correspondence
Nicola Decaro
nicola.decaro@uniba.it

Received 25 May 2012
Accepted 28 June 2012

A Hobi-like pestivirus pair consisting of cytopathogenic (cp) and non-cytopathogenic (noncp) strains, Italy 83/10cp and Italy 83/10ncp, was isolated from the lung of a heifer that died of respiratory disease. The noncp and cp viruses were isolated on Madin–Darby bovine kidney cells and separated by plaque purification and end point dilution. Analysis of the nearly full-length genomes revealed that the two viruses were very closely related to each other and to the noncp Hobi-like strain Italy 1/10-1, which had been isolated a few weeks earlier from the same herd. One major difference between noncp and cp viruses concerned the presence of a cellular Jiv sequence in the 3′ domain of the NS2-encoding region of the cp strain. This is the first study, to our knowledge, reporting the isolation and molecular characterization of a Hobi-like virus pair.

INTRODUCTION

Together with *classical swine fever virus* (CSFV), *border disease virus* (BDV) and other pestiviruses isolated from wild ruminants, *bovine viral diarrhea virus* (BVDV) belongs to the genus *Pestivirus* of the family *Flaviviridae* (Avalos-Ramirez *et al.*, 2001; Becher *et al.*, 2003; Simmonds *et al.*, 2011). BVDV is an enveloped, single-stranded, positive-sense RNA virus that is responsible for multiple clinical signs (Lindenbach *et al.*, 2007) and there are two main species, termed BVDV-1 and BVDV-2 (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994; Simmonds *et al.*, 2011), with several subtypes within each species (Baule *et al.*, 1997; Wolfmeyer *et al.*, 1997; Becher *et al.*, 1999; Vilcek *et al.*, 1999a, b, c; Couvreur *et al.*, 2002; Flores *et al.*, 2002; Tajima, 2004). The BVDV genomic RNA, approximately 12.3 kb in size, encodes a polyprotein (NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-3-NS4A-NS4B-NS5A-NS5B-COOH) that is processed by viral and cellular proteases, thereby generating structural and non-structural proteins. The single large ORF is flanked by the 5′ and 3′ UTRs (Lindenbach *et al.*, 2007).

An atypical pestivirus, named D32/00_Hobi, was isolated from a contaminated batch of FCS originating from Brazil (Schirrmeier *et al.*, 2004). Hobi-like pestiviruses contaminating FCS of South American origin were later detected in Switzerland (Stalder *et al.*, 2005), Sweden (Liu *et al.*, 2009a), Italy (Peletto *et al.*, 2012), USA, Canada, Mexico and Australia (Xia *et al.*, 2011). Strains responsible for natural infections were recovered in South America and Thailand from bubaline blood (Stalder *et al.*, 2005) and bovine serum (Ståhl *et al.*, 2007), but there was no evidence of associated clinical signs. All these viruses were proposed to belong to a new pestivirus species, tentatively termed BVDV-3 (Liu *et al.*, 2009b). However, there is no agreement among pestivirologists about this proposal, considering the genetic and antigenic distance of the new viruses from other BVD viruses (J. Ridpath, personal communication). The first European Hobi-like virus strain Italy 1/10-1 was isolated from calves with severe respiratory disease in southern Italy (Decaro *et al.*, 2011). This is also the first report, to our knowledge, describing the occurrence of disease associated with this novel pestivirus group. Experimental infection of cattle, sheep and swine showed that the Hobi-like pestivirus is able to infect all those species, although only ruminants displayed clinical signs and virus shedding (Decaro *et al.*, 2012b). Additional Hobi-like viruses were associated with abortion in multiparous cows of the same herd (Decaro *et al.*, 2012a) and with respiratory disease in cattle of a neighbouring region in Italy (Decaro *et al.*, 2012c).

On the basis of the capacity to cause a cytopathic effect in the infected cell cultures, two BVDV biotypes are known, cytopathogenic (cp) and non-cytopathogenic (noncp); both are involved in the pathogenesis of mucosal disease (MD), a fatal outcome of BVDV infection in persistently infected calves (Brownlie *et al.*, 1984; Bolin *et al.*, 1985). Typically, an animal affected by MD is infected by both cp and noncp virus, which are called a virus pair (Meyers & Thiel, 1996). Extensive molecular analyses have demonstrated that cp

The GenBank/EMBL/DDBJ accession numbers for the complete genomes of Hobi-like pestivirus strains Italy 83/10ncp and Italy 83/10cp are JQ612704 and JQ612705, respectively.

BVDV is derived from noncp BVDV by various mutations, which consist of insertions of cellular sequences, duplications or deletions of viral sequences (Meyers & Thiel, 1996; Ridpath & Neill, 2000; Neill & Ridpath, 2001), or point mutations within the NS2 gene (Kümmerer *et al.*, 1998). All these changes lead to the production of large amounts of free NS3 protein, which apparently represents an apoptotic signal for the infected cells (Donis & Dubovi, 1987; Pocock *et al.*, 1987).

cp strains have been reported for different pestivirus species, including BVDV-1, BVDV-2, BDV, CSFV and pestivirus of giraffe, while to date, only noncp strains have been described for Hobi-like pestiviruses (Schirrmeier *et al.*, 2004; Ståhl *et al.*, 2007; Decaro *et al.*, 2011, 2012a). In the present study, we report the genetic characterization of the first Hobi-like cp strain isolated from a heifer that died as a consequence of respiratory distress.

RESULTS

Respiratory distress in a heifer, with known exposure of the cattle herd to Hobi-like pestivirus

An outbreak of Hobi-like-induced respiratory disease occurred in a cattle herd in the Calabria region, southern Italy, between December 2009 and February 2010, involving 26 6–7-month-old calves, two of which died (Decaro *et al.*, 2011). In March 2010, a 13-month-old heifer (Prot. No. 83/10) died 6 days after displaying respiratory disease characterized by anorexia, fever (40.3 °C), moderate nasal discharge, dry coughing, open mouth, stretched neck and extended forelimbs. Haematological investigations showed acute leukopenia (2.87×10^9 cells l^{-1}, reference range 4.00–12.00×10^9 cells l^{-1}). Upon necropsy, only gross lesions of the respiratory tract (catarrhal bronchopneumonia and tracheitis) were evident. Lung tissue samples were collected for laboratory investigations and they tested positive for the ruminant pestivirus E^{rns} gene (Sullivan & Akkina, 1995). Other viral, bacterial and parasitic pathogens of cattle, including other respiratory viruses, *Mannheimia* spp., *Pasteurella* spp., *Haemophilus* spp. and pulmonary strongyles, were not detected in the examined samples.

Identification and purification of a Hobi-like virus pair

A BLAST search revealed that the sequence of the E^{rns} fragment amplified by PCR was related to BVDV-2 USA strains p11Q and 296nc with about 74 % nucleotide identity. In contrast, a 100 % nucleotide identity was displayed to the Hobi-like pestivirus strain Italy 1/10-1 that had been detected in the same herd. The Hobi-like sequence was then confirmed by a recently established nested PCR assay for the typing of bovine pestiviruses (Decaro *et al.*, 2012c).

A real-time RT-PCR assay specific for Hobi-like viruses was carried out for the detection and quantification of the viral RNA in the lungs, which were found to contain 7.98×10^6 copies of viral RNA (μl template)$^{-1}$.

Inoculated Madin–Darby bovine kidney (MDBK) cells displayed cytoplasmic fluorescence in an immunofluorescence (IF) assay using a pestivirus mAb (Fig. 1a, b). Morphological changes characteristic for cp pestiviral replication were observed in infected cell cultures that consisted of cytoplasmic vacuolization, cell rounding and detachment from the monolayer (Fig. 1c, d).

In order to cover the nearly full-length genome of the atypical pestivirus, overlapping regions were amplified by RT-PCR from the original lung samples. After gel electrophoresis, two clearly distinct bands were visible in the RT-PCR product that was obtained by using primers to amplify a genomic region encompassing the NS2–3 gene, whereas the Italian prototype atypical strain yielded a single band corresponding to the smaller amplicon of the new Hobi-like strain (Fig. 2a). Sequence analysis of the two bands excised from the electrophoretic gel showed a 79.7 % nucleotide identity due to the presence of a 306 nt insert in the larger amplicon.

The cp virus (Italy 83/10cp) was purified by three successive plaque pickings, whereas the noncp isolate (Italy 83/10ncp) was separated by serial passage at limiting dilutions.

Molecular characterization of the Hobi-like virus pair

The sequences of the near full-length genomes of the BVD virus pair consisting of Italy 83/10ncp and Italy 83/10cp were determined by RT-PCR amplifications and subsequent sequencing of the overlapping fragments obtained from the purified viruses. The obtained nucleotide sequences were deposited in GenBank under accession numbers JQ612704 and JQ612705, respectively. By comparison with reference sequences, strains Italy 83/10cp and Italy 83/10ncp were found to have the same genomic organization as other members of the genus *Pestivirus*, consisting of a unique ORF of 11 700 (noncp strain) or 12 006 (cp strain) nt flanked by two UTRs. The ORFs encode 11 proteins with the same size as determined for Hobi-like prototype strains Italy 1/10-1 and Th/04_KhonKaen, with the exception of the NS2–3 product which was 102 aa longer in the cp virus.

By sequence analysis, the pestivirus pair exhibited 97.0 % nucleotide identity, differing mainly because of the presence of the Jiv insertion in the cp virus genome. This identity reached 99.5 % when the insertion was removed. The nucleotide identities to the genome of the atypical pestivirus previously detected in the same herd (strain Italy 1/10-1) were 99.0 and 96.5 % for noncp and cp strains, respectively. Apart from the cellular insert present in strain Italy 83/10cp, only point mutations located throughout the genome were detected in both cp and noncp viruses in comparison with Hobi-like strain Italy 1/10-1. The insertion detected in the cp strain was highly similar

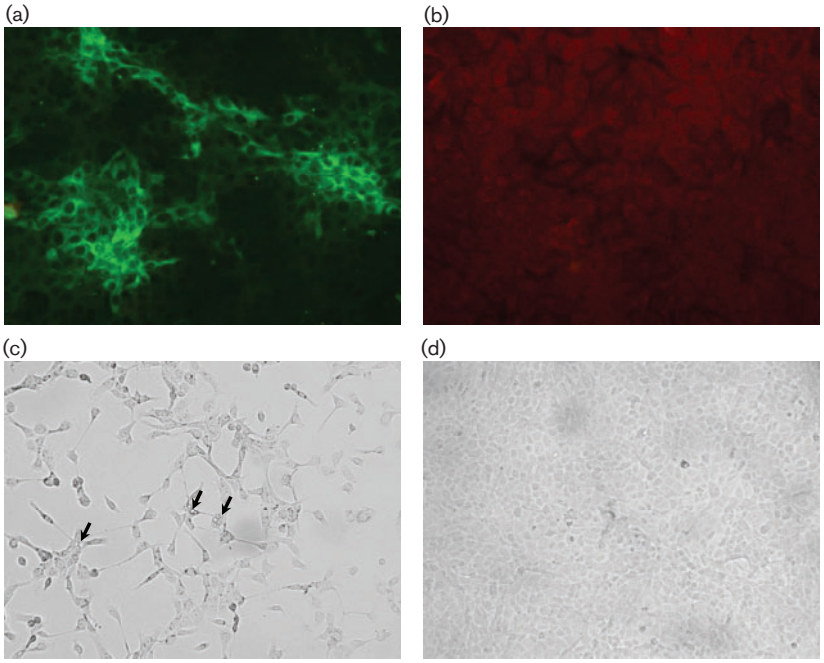

Fig. 1. (a) Cytoplasmic fluorescence detected by the IF assay using an NS3 mAb. (b) Mock-inoculated cells submitted to the IF assay. (c) Cytopathic effect consisting of cytoplasmic vacuolization (arrows) and lysis of the infected monolayer. (d) Mock-inoculated cells. Magnification: ×400 (a,b) and ×100 (c,d).

(98.6 % nucleotide identity) to a *Bos taurus* cellular sequence (GenBank accession no. AY027882) of the J-domain family, termed J-domain protein interacting with viral protein (Jiv), that was recognized to be involved in the shift from noncp to cp pestivirus biotype (Becher *et al.*, 1996). By comparative analysis with 14 cp pestiviruses displaying Jiv sequences, including two BDV, three BVDV-1, eight BVDV-2 and one pestivirus of giraffe strains, the cellular insert of the Hobi-like cp strain (Italy 83/10cp) was 102 aa and was located at the 3′ end of the NS2 gene, at position A of the pestivirus genome that corresponds to the residue 1535 of the BVDV-1 strain SD1 polyprotein (Meyers *et al.*, 1998). There were two amino acid changes in the Italy 83/10cp insert with respect to the cellular Jiv, one of which was unique to the isolated pestivirus strain (Fig. 2b).

Using the neighbour-joining method, a phylogenetic tree was inferred from the full-length genomic sequences. The analysed pestiviruses clustered into six monophyletic clades consisting of BVDV-1, BVDV-2, Hobi-like pestiviruses, BDV, CSFV and the pestivirus of giraffe (Fig. 3). In this tree, strains Italy 83/10cp and Italy 83/10ncp formed a unique cluster with atypical pestivirus prototype strains Italy 1/10-1 and Th/04_KhonKaen, which was clearly separated from the clusters of the other pestivirus species. This pattern of segregation was confirmed by the maximum-parsimony method (data not shown).

DISCUSSION

Different mutations have been associated with the emergence of cp BVDVs, which mostly concerned the genomic region encoding NS2–3 and resulted in the generation of enhanced levels of NS3 (Meyers & Thiel, 1996; Peterhans *et al.*, 2010). In many instances, NS3 is produced by cleavage of the NS2–3 complex as a consequence of non-homologous recombination with host-cell RNA sequences. One of the most frequent genomic changes is the insertion of ubiquitin-coding sequences right upstream of the NS3 gene. In the translated polyprotein, the NS2–ubiquitin fusion protein is cleaved by cellular proteases; this leads to efficient release of NS3 (Tautz *et al.*, 1993). An alternative way associated with generation of free NS3 is the insertion of Jiv sequences of host-cell origin within NS2 upstream of the NS2–3 cleavage site (Ridpath & Neill, 2000; Vilcek *et al.*, 2000; Neill & Ridpath, 2001; Becher & Tautz, 2011). Jiv is a member of the J-domain protein family, a group of cellular chaperones interacting with Hsp70 and implicated in virus replication (Hu *et al.*, 1997; Tomita *et al.*, 2003). For efficient induction of NS2–3 cleavage, a 90 aa fragment of Jiv, termed Jiv90, was shown to be essential (Rinck *et al.*, 2001). Further studies have shown that Jiv acts as a co-factor, stimulating a conformational change in the NS2–3 complex with subsequent activation of the pestiviral NS2 autoprotease (Lackner *et al.*, 2005). Indeed, cellular Jiv is also essential for the replication of noncp BVDV, by stimulation of active cleavage of NS2–3 *in trans* and the release of a small amount of free NS3, which is an essential component of the viral replicase complex (Lackner *et al.*, 2004). In noncp BVDV, generation of NS3 is down-regulated by the limited availability of intracellular Jiv, leading to severe restriction of viral replication and establishment of life-long persistent infections in calves infected transplacentally (Lackner *et al.*, 2005). In contrast, Jiv overexpression due to the incorporation of Jiv sequences into the 3′ region of the NS2-encoding region

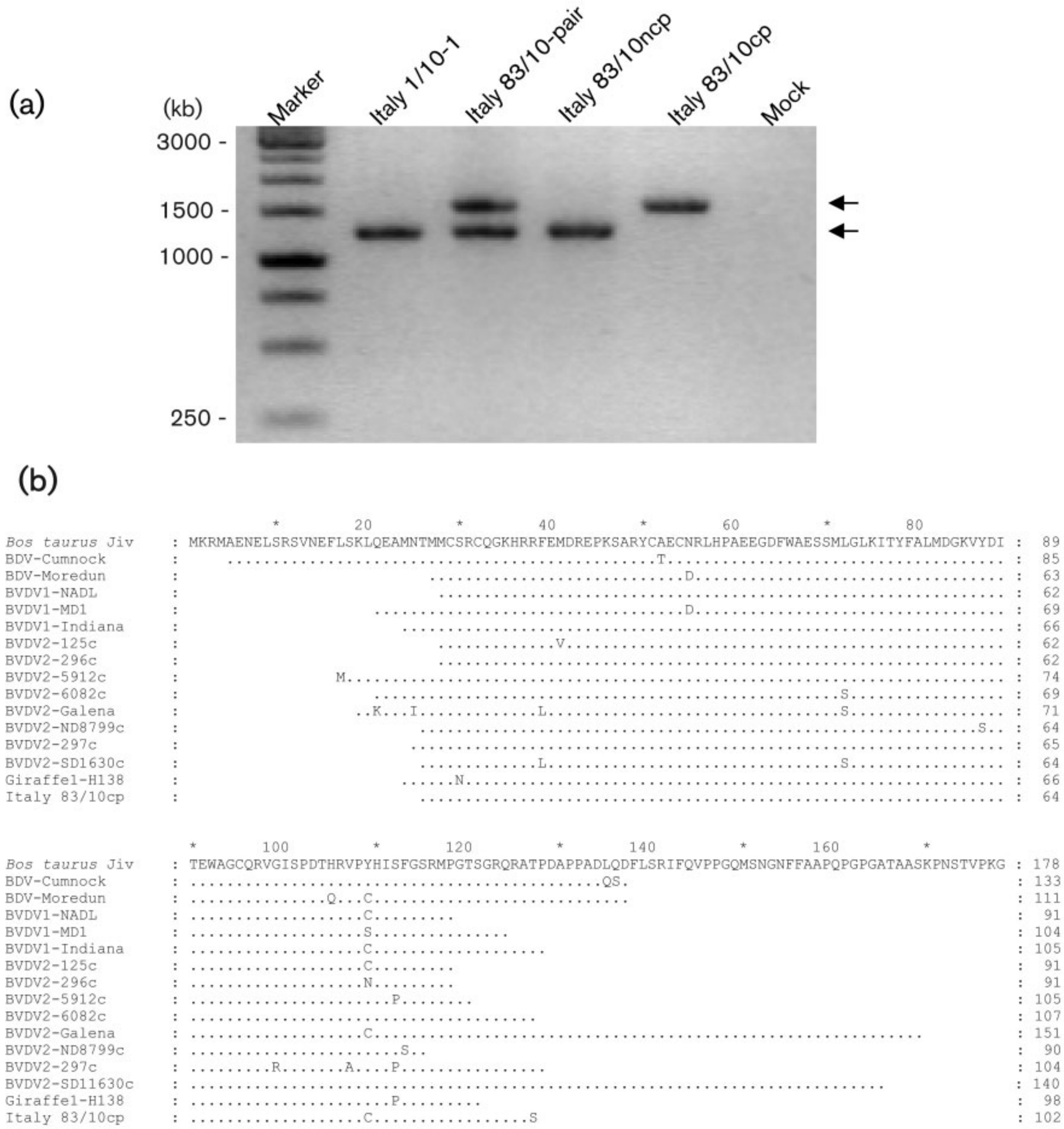

Fig. 2. Molecular characterization of the cytopathogenicity of strain Italy 83/10cp. (a) RT-PCR amplification of a genomic region encompassing the NS2–3-coding sequence. Lanes: Marker, GeneRuler 1 kb DNA Ladder (MBI Fermentas); Italy 1/10-1, MDBK cells infected with Italian noncp BVDV-3 strain (Decaro *et al.*, 2011); Italy 83/10-pair, lung sample of the dead heifer; Italy 83/10ncp, MDBK cells infected with the purified noncp strain; Italy 83/10cp, MDBK cells infected with the purified cp strain; Mock, uninfected cells. Arrows indicate amplicons obtained from cp (larger band) and noncp (smaller band) strains, respectively. (b) Comparison of Jiv inserts from 15 pestiviruses. The amino acid sequence of the portion of the Jiv inserts found within the NS2–3-coding region of the 15 pestiviruses was compared with the cellular Jiv sequence (top sequence, GenBank accession no. AY027882). Dots indicate conserved residues with respect to the cellular Jiv. The following pestivirus strains were aligned (GenBank nucleotide accession nos are reported in parentheses): BDV strains: Cumnock (U43603), Moredun (U43602); BVDV-1 strains: NADL (AJ133738), MD1 (Z54332), Indiana (Z54331); BVDV-2 strains: 125c (U25053), 296c (AF268172), 5912c (AF268179), 6082c (AF268180), Galena (AF268176), ND8799c (AF268175); 297c (AF268177), SD1630c (AF268178); pestivirus of giraffe 1-H138 (AF144617); Hobi-like pestivirus Italy 83/10cp (JQ612705).

upregulates the generation of NS3 and thereby enhances viral replication through production of major amounts of active replicase complexes (Lackner *et al.*, 2005). Jiv sequences have been detected in the NS2-encoding region of BDV, few BVDV-1 and several BVDV-2 cp strains (Neill and Ridpath, 2001), as well as in that of the prototype of

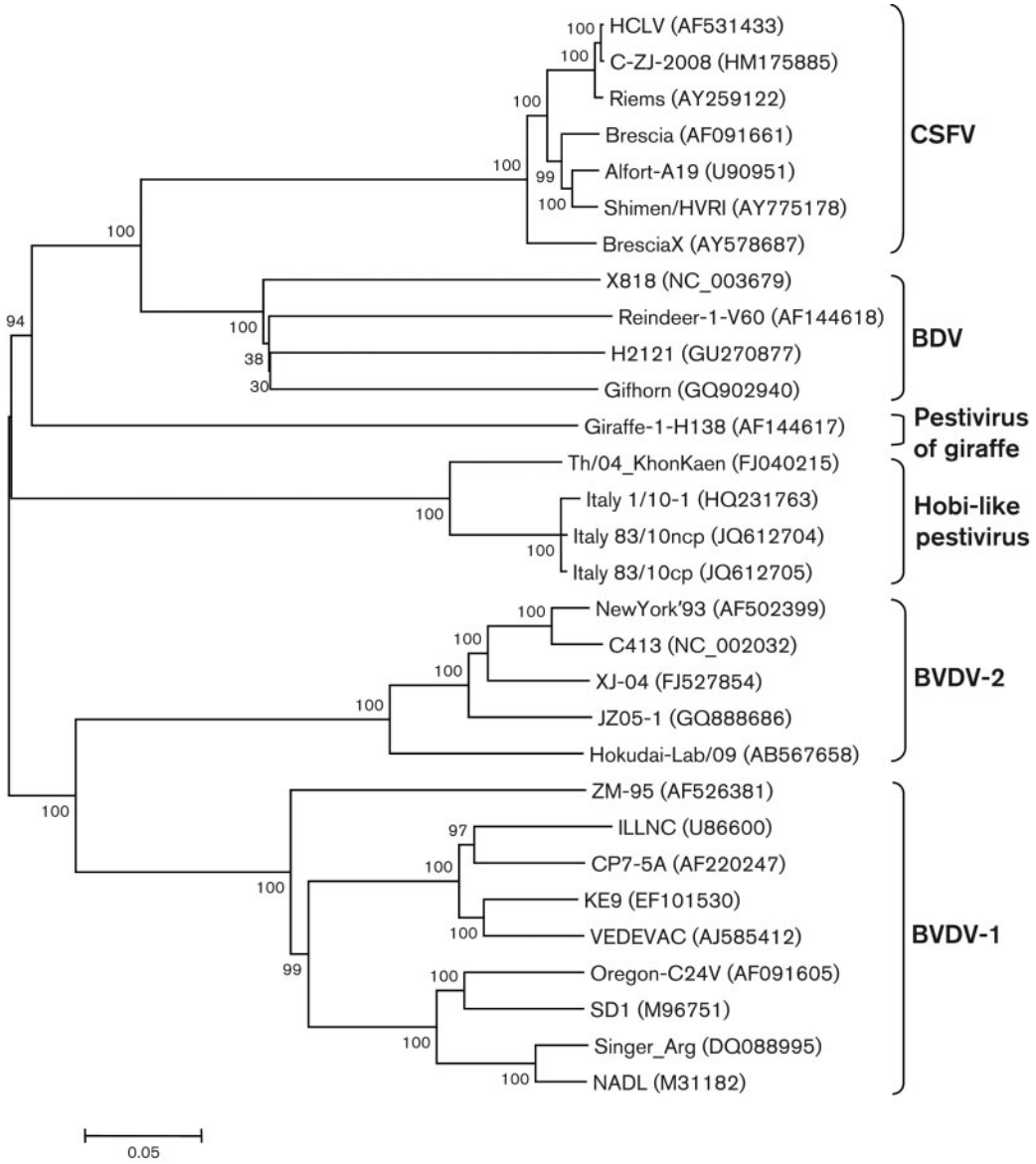

Fig. 3. Neighbour-joining tree based on the full-length genome of members of the genus *Pestivirus*. For the phylogenetic tree construction, the following pestivirus species were used (GenBank accession nos are reported in parentheses): BDV strains: H2121, Gifhorn, X818, Reindeer-1-V60; CSFV strains: BresciaX, HCLV, Brescia, Alfort-A19, Shimen/HVRI, Riems, C-ZJ-2008; BVDV-1 strains: ILLNC, ZM-95, Oregon-C24V, CP7-5A, SD1, Singer_Arg, KE9, NADL, VEDEVAC; BVDV-2 strains: JZ05-1, New York'93, XJ-04, C413, Hokudai-Lab/09; Hobi-like strains: Th/04_KhonKaen, Italy 1/10-1, Italy 83/10ncp, Italy 83/10cp; pestivirus of giraffe 1-H138. Statistical support was provided by bootstrapping over 1000 replicates. Bar, 0.05 nucleotide substitutions per site.

the tentative pestiviral species, pestivirus of giraffe, strain Giraffe-1 H138 (Avalos-Ramirez *et al.*, 2001).

After the first isolation of the atypical pestivirus D32/00_Hobi from contaminated FCS in Brazil (Schirrmeier *et al.*, 2004), Hobi-like pestiviruses have been detected in FCS batches, as well as in ruminants with natural infections in several countries (Stalder *et al.*, 2005; Ståhl *et al.*, 2007; Liu *et al.*, 2009a; Xia *et al.*, 2011; Peletto *et al.*, 2012; Decaro *et al.*, 2011, 2012a). Apart from the Italian Hobi-like strains, atypical pestiviruses have never been associated with the occurrence of clinical signs (Decaro *et al.*, 2011, 2012a). In addition, all strains characterized so far were noncp viruses. Thus, the present study is the first reporting the isolation and genetic characterization of a cp Hobi-like strain. The respective virus pair (cp and noncp strain) was isolated from the lungs of a dead heifer, which displayed severe respiratory disease. The extremely high genetic

relatedness between the two viruses (which diverged only in the NS2–3-coding region) strongly suggests that strain Italy 83/10cp is derived from strain Italy 83/10ncp through heterologous recombination with Jiv sequences of cellular origin.

BVDV pairs are usually isolated from cattle that developed the highly lethal MD. According to current knowledge, MD occurs in animals which are persistently infected *in utero* with noncp BVDV. Such animals are immunotolerant with respect to the persisting virus strain and may spontaneously come down with MD. This disease is characterized by haemorrhagic, necrotic and ulcerative lesions. It is puzzling that the Hobi-like virus pair described in this study was derived from a heifer that died of respiratory disease, whereas classical signs and gross lesions of MD were not observed. However, in view of the fact that clinical signs of both acute and persistent infection with BVDV may be extremely diverse, the signs observed in this animal may be unique and do not necessarily indicate a major difference between the Hobi-like and classical pestiviruses. It could not be shown that the diseased animal was persistently infected with noncp BVDV. It remains to be demonstrated that the cp virus is responsible for the observed clinical signs and gross lesions of the respiratory tract. These open questions will be addressed by follow-up studies of field cases and by animal experiments. It is certainly worthwhile to determine whether the pathogenic potential of Hobi-like viruses can differ from that of the established BVDV species.

METHODS

Molecular investigations. Lung samples of the dead heifer were subjected to molecular assays for the detection of the main respiratory viruses of cattle. Nucleic acids for RT-PCR assays were purified using the commercial kits DNeasy tissue kit (Qiagen), QIAamp viral RNA mini kit (Qiagen) (nasal swabs) or QIAamp RNeasy mini kit (Qiagen) (lung samples).

RNA extracts were used for the detection of bovine coronavirus (Decaro *et al.*, 2008), bovine parainfluenza virus type 3 (Lyon *et al.*, 1997), bovine pestiviruses (Sullivan & Akkina, 1995; Decaro *et al.*, 2012c) and bovine respiratory syncytial virus (Valarcher *et al.*, 1999). Detection of bovine herpesvirus types 1 (Vilcek, 1993) and 4 (Boerner *et al.*, 1999) was carried out on the DNA templates. RT-PCR and PCR assays were performed using SuperScript One-Step RT-PCR for Long Templates (Life Technologies) and LA PCR kit Ver. 2.1 (TaKaRa Bio), respectively.

Bacteriological and parasitological investigations. Standardized procedures were carried out on lung samples for *in vitro* isolation of the most common bacteria responsible for respiratory disease in cattle. Samples were plated out on 5 % sheep blood agar and cultured aerobically at 37 °C for 24 h for the detection of aerobic pathogens. Bacteria were identified by standard biochemical procedures and analytical profile index (bioMérieux). For isolation of mycoplasmas, samples were inoculated into modified Hayflick broth at 37 °C, as previously described (Buonavoglia *et al.*, 2008, 2010). Detection of the most common respiratory parasites was achieved by faecal flotation.

Real-time RT-PCR for Hobi-like pestiviruses. A real-time RT-PCR assay based on TaqMan technology (Liu *et al.*, 2008) was used to detect the atypical pestivirus and quantify the viral load in samples that tested positive for bovine pestiviruses (Sullivan & Akkina, 1995; Decaro *et al.*, 2012c). Hobi-like RNA copy numbers were calculated on the basis of two standard curves generated by 10-fold dilutions of synthetic RNA obtained by *in vitro* transcription of a plasmid containing the 5′ UTR of strain Italy 1/10-1. Reverse transcription was carried out using GeneAmp RNA PCR (Applied Biosystems), following the manufacturer's recommendations. The quantitative assay targeting the 5′ UTR was conducted in a 50 µl reaction mixture containing 25 µl IQ Supermix (Bio-Rad Laboratories), 600 nM primers T134-F (5′-GACTAGTGGTGGCAGTGAGC-3′) and T220-R (5′-GAGGCATTCCTTGATGCGTC-3′), 200 nM probe T155r-P (6FAM-5′-ACTCGGGGCTTCGGTGATCCAGGG-3′-BHQ1) and 20 µl cDNA. The thermal profile consisted of activation of iTaq DNA polymerase at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min.

Pestivirus pair isolation and purification. Virus isolation was carried out on the lung samples collected from the dead heifer. Tissue samples were homogenized in Dulbecco's minimal essential medium containing antibiotics (5000 IU penicillin ml^{-1}, 2500 µg streptomycin ml^{-1}, 10 µg amphotericin B ml^{-1}). After centrifugation at 3000 ***g*** for 15 min, the supernatant was used to inoculate confluent monolayers of MDBK cells. Viral growth was monitored by an IF assay using a BVDV mAb and a goat anti-mouse IgG conjugated with fluorescein isothiocyanate (Sigma-Aldrich).

Cp and noncp Hobi-like viruses were separated through successive plaque and end point dilution assays, respectively, as described by Ridpath *et al.* (1991).

Genomic analysis of the Hobi-like virus pair. The near full-length genome sequences of strains Italy 83/10cp and Italy 83/10ncp were determined from the purified viral isolates by means of RT-PCR amplifications carried out as described by Liu *et al.* (2009c) using the SuperScript One-Step RT-PCR for Long Templates (Life Technologies). The PCR products generated from the atypical pestivirus were directly sequenced by BaseClear B.V. (Leiden, The Netherlands) and a consensus sequence was obtained for each virus using the CAP option of the BioEdit software package (Hall, 1999). Additional RT-PCR assays and sequencing attempts were performed to close gaps between assembled contigs using strain-specific primers. The consensus nucleotide sequences were manually refined with GeneDoc (http://www.nrbsc.org/gfx/genedoc/) and aligned using the CLUSTAL W tool of the European Molecular Biology Laboratory (http://www.ebi.ac.uk). The nearly full-length sequences of cp and noncp viruses were compared to the analogous sequences of pestivirus cp and noncp reference strains, including the Italian prototype Hobi-like strain (Decaro *et al.*, 2011). The same sequences were subjected to phylogenetic analysis using both parsimony and neighbour-joining methods of the MEGA4.1 software (Tamura *et al.*, 2007), supplying statistical support with bootstrapping over 1000 replicates.

ACKNOWLEDGEMENTS

This work was supported by grants from the University of Bari, Italy, Ricerca di Ateneo 2010, project Epidemiologia dei pestivirus atipici (BVDV-3) in Italia and from the Italian Ministry of Health, Ricerca corrente 2009, project Epidemiologia del virus della diarrea virale bovina tipo 3 (BVDV-3) nel Sud Italia.

REFERENCES

Avalos-Ramirez, R., Orlich, M., Thiel, H. J. & Becher, P. (2001). Evidence for the presence of two novel pestivirus species. *Virology* **286**, 456–465.

Baule, C., van Vuuren, M., Lowings, J. P. & Belák, S. (1997). Genetic heterogeneity of bovine viral diarrhoea viruses isolated in Southern Africa. *Virus Res* **52**, 205–220.

Becher, P. & Tautz, N. (2011). RNA recombination in pestiviruses: cellular RNA sequences in viral genomes highlight the role of host factors for viral persistence and lethal disease. *RNA Biol* **8**, 216–224.

Becher, P., Meyers, G., Shannon, A. D. & Thiel, H. J. (1996). Cytopathogenicity of border disease virus is correlated with integration of cellular sequences into the viral genome. *J Virol* **70**, 2992–2998.

Becher, P., Orlich, M., König, M. & Thiel, H. J. (1999). Nonhomologous RNA recombination in bovine viral diarrhea virus: molecular characterization of a variety of subgenomic RNAs isolated during an outbreak of fatal mucosal disease. *J Virol* **73**, 5646–5653.

Becher, P., Avalos Ramirez, R., Orlich, M., Cedillo Rosales, S., König, M., Schweizer, M., Stalder, H., Schirrmeier, H. & Thiel, H. J. (2003). Genetic and antigenic characterization of novel pestivirus genotypes: implications for classification. *Virology* **311**, 96–104.

Boerner, B., Weigelt, W., Buhk, H. J., Castrucci, G. & Ludwig, H. (1999). A sensitive and specific PCR/Southern blot assay for detection of bovine herpesvirus 4 in calves infected experimentally. *J Virol Methods* **83**, 169–180.

Bolin, S. R., McClurkin, A. W., Cutlip, R. C. & Coria, M. F. (1985). Severe clinical disease induced in cattle persistently infected with noncytopathic bovine viral diarrhea virus by superinfection with cytopathic bovine viral diarrhea virus. *Am J Vet Res* **46**, 573–576.

Brownlie, J., Clarke, M. C. & Howard, C. J. (1984). Experimental production of fatal mucosal disease in cattle. *Vet Rec* **114**, 535–536.

Buonavoglia, D., Greco, G., Quaranta, V., Corrente, M., Martella, V. & Decaro, N. (2008). An oil-emulsion vaccine induces full-protection against *Mycoplasma agalactiae* infection in sheep. *New Microbiol* **31**, 117–123.

Buonavoglia, D., Greco, G., Corrente, M., Greco, M. F., D'Abramo, M., Latronico, F., Fasanella, A. & Decaro, N. (2010). Long-term immunogenicity and protection against *Mycoplasma agalactiae* induced by an oil adjuvant vaccine in sheep. *Res Vet Sci* **88**, 16–19.

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.

Decaro, N., Elia, G., Campolo, M., Desario, C., Mari, V., Radogna, A., Colaianni, M. L., Cirone, F., Tempesta, M. & Buonavoglia, C. (2008). Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. *J Virol Methods* **151**, 167–171.

Decaro, N., Lucente, M. S., Mari, V., Cirone, F., Cordioli, P., Camero, M., Sciarretta, R., Losurdo, M., Lorusso, E. & Buonavoglia, C. (2011). Atypical pestivirus and severe respiratory disease in calves, Europe. *Emerg Infect Dis* **17**, 1549–1552.

Decaro, N., Lucente, M. S., Mari, V., Sciarretta, R., Pinto, P., Buonavoglia, D., Martella, V. & Buonavoglia, C. (2012a). Hobi-like pestivirus in aborted bovine fetuses. *J Clin Microbiol* **50**, 509–512.

Decaro, N., Mari, V., Lucente, M. S., Sciarretta, R., Moreno, A., Armenise, C., Losurdo, M., Camero, M., Lorusso, E. & other authors (2012b). Experimental infection of cattle, sheep and pigs with 'Hobi'-like pestivirus. *Vet Microbiol* **155**, 165–171.

Decaro, N., Sciarretta, R., Lucente, M. S., Mari, V., Amorisco, F., Colaianni, M. L., Cordioli, P., Parisi, A., Lelli, R. & Buonavoglia, C. (2012c). A nested PCR approach for unambiguous typing of pestiviruses infecting cattle. *Mol Cell Probes* **26**, 42–46.

Donis, R. O. & Dubovi, E. J. (1987). Differences in virus-induced polypeptides in cells infected by cytopathic and noncytopathic biotypes of bovine virus diarrhea-mucosal disease virus. *Virology* **158**, 168–173.

Flores, E. F., Ridpath, J. F., Weiblen, R., Vogel, F. S. & Gil, L. H. (2002). Phylogenetic analysis of Brazilian bovine viral diarrhea virus type 2 (BVDV-2) isolates: evidence for a subgenotype within BVDV-2. *Virus Res* **87**, 51–60.

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

Hu, J., Toft, D. O. & Seeger, C. (1997). Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO J* **16**, 59–68.

Kümmerer, B. M., Stoll, D. & Meyers, G. (1998). Bovine viral diarrhea virus strain Oregon: a novel mechanism for processing of NS2-3 based on point mutations. *J Virol* **72**, 4127–4138.

Lackner, T., Müller, A., Pankraz, A., Becher, P., Thiel, H. J., Gorbalenya, A. E. & Tautz, N. (2004). Temporal modulation of an autoprotease is crucial for replication and pathogenicity of an RNA virus. *J Virol* **78**, 10765–10775.

Lackner, T., Müller, A., König, M., Thiel, H. J. & Tautz, N. (2005). Persistence of bovine viral diarrhea virus is determined by a cellular cofactor of a viral autoprotease. *J Virol* **79**, 9746–9755.

Lindenbach, B. D., Thiel, H.-J. & Rice, C. M. (2007). Flaviviridae: the viruses and their replication. In *Fields Virology*, 5th edn, vol. 1, pp. 1101–1152. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.

Liu, L., Xia, H., Belák, S. & Baule, C. (2008). A TaqMan real-time RT-PCR assay for selective detection of atypical bovine pestiviruses in clinical samples and biological products. *J Virol Methods* **154**, 82–85.

Liu, L., Kampa, J., Belák, S. & Baule, C. (2009a). Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen. *Vet Microbiol* **138**, 62–68.

Liu, L., Xia, H., Baule, C. & Belák, S. (2009b). Maximum likelihood and Bayesian analyses of a combined nucleotide sequence dataset for genetic characterization of a novel pestivirus, SVA/cont-08. *Arch Virol* **154**, 1111–1116.

Liu, L., Xia, H., Wahlberg, N., Belák, S. & Baule, C. (2009c). Phylogeny, classification and evolutionary insights into pestiviruses. *Virology* **385**, 351–357.

Lyon, M., Leroux, C., Greenland, T., Chastang, J., Patet, J. & Mornex, J. F. (1997). Presence of a unique parainfluenza virus 3 strain identified by RT-PCR in visna-maedi virus infected sheep. *Vet Microbiol* **57**, 95–104.

Meyers, G. & Thiel, H. J. (1996). Molecular characterization of pestiviruses. *Adv Virus Res* **47**, 53–118.

Meyers, G., Stoll, D. & Gunn, M. (1998). Insertion of a sequence encoding light chain 3 of microtubule-associated proteins 1A and 1B in a pestivirus genome: connection with virus cytopathogenicity and induction of lethal disease in cattle. *J Virol* **72**, 4139–4148.

Neill, J. D. & Ridpath, J. F. (2001). Recombination with a cellular mRNA encoding a novel DnaJ protein results in biotype conversion in genotype 2 bovine viral diarrhea viruses. *Virus Res* **79**, 59–69.

Peletto, S., Zuccon, F., Pitti, M., Gobbi, E., Marco, L. D., Caramelli, M., Masoero, L. & Acutis, P. L. (2012). Detection and phylogenetic analysis of an atypical pestivirus, strain IZSPLV_To. *Res Vet Sci* **92**, 147–150.

Pellerin, C., van den Hurk, J., Lecomte, J. & Tijssen, P. (1994). Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. *Virology* **203**, 260–268.

Peterhans, E., Bachofen, C., Stalder, H. & Schweizer, M. (2010). Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. *Vet Res* **41**, 44.

Pocock, D. H., Howard, C. J., Clarke, M. C. & Brownlie, J. (1987). Variation in the intracellular polypeptide profiles from different isolates of bovine virus diarrhoea virus. *Arch Virol* **94**, 43–53.

Ridpath, J. F. & Neill, J. D. (2000). Detection and characterization of genetic recombination in cytopathic type 2 bovine viral diarrhea viruses. *J Virol* **74**, 8771–8774.

Ridpath, J. F., Lewis, T. L., Bolin, S. R. & Berry, E. S. (1991). Antigenic and genomic comparison between non-cytopathic and cytopathic bovine viral diarrhoea viruses isolated from cattle that had spontaneous mucosal disease. *J Gen Virol* **72**, 725–729.

Ridpath, J. F., Bolin, S. R. & Dubovi, E. J. (1994). Segregation of bovine viral diarrhea virus into genotypes. *Virology* **205**, 66–74.

Rinck, G., Birghan, C., Harada, T., Meyers, G., Thiel, H. J. & Tautz, N. (2001). A cellular J-domain protein modulates polyprotein processing and cytopathogenicity of a pestivirus. *J Virol* **75**, 9470–9482.

Schirrmeier, H., Strebelow, G., Depner, K., Hoffmann, B. & Beer, M. (2004). Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. *J Gen Virol* **85**, 3647–3652.

Simmonds, P., Becher, P., Collet, M. S., Gould, E. A., Heinz, F. X., Meyers, G., Monath, T., Pletnev, A., Rice, C. M. & other authors (2011). *Family Flaviviridae. Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses*, pp. 1003–1020. Edited by A. M. Q. King, M. J. Adams, E. B. Carstens & E. J. Lefkowitz. Amsterdam: Elsevier Academic Press.

Ståhl, K., Kampa, J., Alenius, S., Persson Wadman, A., Baule, C., Aiumlamai, S. & Belák, S. (2007). Natural infection of cattle with an atypical 'HoBi'-like pestivirus – implications for BVD control and for the safety of biological products. *Vet Res* **38**, 517–523.

Stalder, H. P., Meier, P., Pfaffen, G., Wageck-Canal, C., Rüfenacht, 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, discussion 215–219.

Sullivan, D. G. & Akkina, R. K. (1995). A nested polymerase chain reaction assay to differentiate pestiviruses. *Virus Res* **38**, 231–239.

Tajima, M. (2004). Bovine viral diarrhea virus 1 is classified into different subgenotypes depending on the analyzed region within the viral genome. *Vet Microbiol* **99**, 131–138.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). mega4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Tautz, N., Meyers, G. & Thiel, H.-J. (1993). Processing of polyubiquitin in the polyprotein of an RNA virus. *Virology* **197**, 74–85.

Tomita, Y., Mizuno, T., Díez, J., Naito, S., Ahlquist, P. & Ishikawa, M. (2003). Mutation of host DnaJ homolog inhibits brome mosaic virus negative-strand RNA synthesis. *J Virol* **77**, 2990–2997.

Valarcher, J. F., Bourhy, H., Gelfi, J. & Schelcher, F. (1999). Evaluation of a nested reverse transcription-PCR assay based on the nucleoprotein gene for diagnosis of spontaneous and experimental bovine respiratory syncytial virus infections. *J Clin Microbiol* **37**, 1858–1862.

Vilcek, S. (1993). Detection of the bovine herpesvirus-1 (BHV-1) genome by PCR. *J Virol Methods* **41**, 245–247.

Vilcek, S., Alenius, S., Paton, D. J., Mittelholzer, C. & Belák, S. (1999a). Genetic clustering of bovine viral diarrhoea viruses in cattle farms: genetic identification and analysis of viruses directly from cattle sera. *Vet J* **158**, 33–38.

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

Vilcek, S., Paton, D., Lowings, P., Björklund, H., Nettleton, P. & Belák, S. (1999c). Genetic analysis of pestiviruses at the 3′ end of the genome. *Virus Genes* **18**, 107–114.

Vilcek, S., Greiser-Wilke, I., Nettleton, P. & Paton, D. J. (2000). Cellular insertions in the NS2-3 genome region of cytopathic bovine viral diarrhoea virus (BVDV) isolates. *Vet Microbiol* **77**, 129–136.

Wolfmeyer, A., Wolf, G., Beer, M., Strube, W., Hehnen, H. R., Schmeer, N. & Kaaden, O. R. (1997). Genomic (5′UTR) and serological differences among German BVDV field isolates. *Arch Virol* **142**, 2049–2057.

Xia, H., Vijayaraghavan, B., Belák, S. & Liu, L. (2011). Detection and identification of the atypical bovine pestiviruses in commercial foetal bovine serum batches. *PLoS ONE* **6**, e28553.