**Detection, genotyping, and antigen distribution of bovine viral diarrhoea virus in archival formalin fixed paraffin embedded tissues**

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**SUMMARY**

This report identifies and genotypes the bovine viral diarrhoea virus (BVDV) in up to 20 years old archival formalin fixed paraffin embedded (FFPE) tissues of bovine foetuses and calves. Samples from 6 presumably BVDV aborted foetuses and 3 calves diagnosed with mucosal disease were tested by immunohistochemistry (IHC), RT-PCR and a one-step multiplex real time RT-PCR for genotyping. Viral RNA was detected in a 20 years old sample from an aborted foetus at which multiple foci of BVDV viral antigen was localized by IHC in the pulmonary tissue. All three calves with mucosal disease were also RT-PCR positive and BVDV-1 was typed using the one-step multiplex real time RT-PCR. The BVDV antigen distribution was demonstrated in the cerebral cortex (in neurons and their processes and in some glial cells), lung, kidneys, oesophagus, tongue, buccal mucosa and ear pinna of one calf affected with mucosal disease. The highest intensity of the antigen signals were seen in the stratum basale and stratum spinosum of the mucosa of the upper digestive tract tissues. The purpose of this study is to further elucidate the diagnosis and genotyping of BVDV infection with respect to prospective and retrospective identification of the virus in routinely processed and stored tissues for histological evaluation.

**Keywords:** Bovine viral diarrhoea virus, antigen distribution, genotyping, formalin fixed paraffin embedded tissues, abortion, mucosal disease.

**Introduction**

Bovine viral diarrhoea virus (BVDV) is a member of the family Flaviviridae, genus Pestivirus. The genus contains four species: BVDV-1, BVDV-2, border disease virus and classical swine fever virus, and each one of them subdivided into several subgenotypes [13]. The viral genome is a positive sense, single stranded, segmented and non-polyadenylated RNA virus that has about 12,300 bases in its genome [9]. Infection with BVDV results in various clinical manifestations in cattle including abortion, congenital defects, foetal mummification, persistent infection, diarrhoea, sudden death, immunosuppression, and mucosal disease complex [6]. This common pathogen of cattle and buffalo is responsible for major economic losses in both the dairy and beef industries globally [14].

Both BVDV-1 and BVDV-2 may exist as two different bio-types, cytopathic (cp) and non cytopathic (ncp), and both species were linked to abortion in cattle [1]. Viral strain and the time of gestation at which the infection occurs determine the consequences of infection which could vary from early embryonic deaths, foetal resorption, mummification, abortion to immunotolerance (90-120 days of gestation with ncp BVDV) accompanied by persistent infection (PI) and congenital defects (100-150 days of gestation) [6, 12]. Only ncp strains of BVDV are associated with PI animals and upon recombination with cellular RNA or superinfection of PI animals with a cp strain, the lethal mucosal disease form develops [16].

Because of the delay between foetal death and subsequent diagnosis of abortion, foetal and placental lesions seen are usually not diagnostic, and virus isolation (VI) is not always successful [3]. Virus isolation is generally quite a sensitive
method of virus detection but the sensitivity is greatly reduced due to autolysis and virus inactivation [2]. Fluorescent antibody technique (FAT) on frozen sections is also used as a routine test in laboratories, although false positive and false negative results were reported when compared to immunohistochemistry (IHC) or VI [7]. Immunohistochemistry on formalin fixed paraffin embedded (FFPE) sections using monoclonal antibody 15C5 is a sensitive and specific method for diagnosis [18]. Reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR for detection of viral nucleic acid from PI calves proved to be highly sensitive both in fresh and FFPE specimens [5]. The purpose of this study is to further elucidate the diagnosis and genotyping of BVDV infection with respect to prospective and retrospective identification of the virus in routinely processed and stored tissues for histological evaluation.

Material and Methods

SAMPLES

Examined samples were archival FFPE tissues from aborted foetuses and mucosal disease confirmed calves that were submitted to the diagnostic pathology laboratory of the Department of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Canada. Cases were selected from 164 necropsied aborted bovine foetuses and mucosal disease calves from 1990 to 2009. The selection was based on the presence of at least one BVDV positive fresh sample during the necropsy by FAT (in old cases) and RT-PCR (in recent cases after 2000).

Among them, a total of 36 FFPE tissue blocks from 6 aborted foetuses and 3 cases of mucosal disease were tested. Age, sex, and necropsy date of the cases are summarized in Table I. Sections of 5 µm were cut, mounted on Super frost/ plus microscope slides (cat #12-550-15, Fisher Sci, USA), and dried overnight at room temperature.

PATHOLOGICAL ANALYSES

Histology

Specimens were examined based on the previously reported clinical history and gross and microscopic lesions. Additional required sections were routinely stained with Haematoxylin-Eosin (HE), examined under microscope and all significant findings were recorded.

Immunohistochemistry

Immunohistochemical tissue BVDV antigen distribution in the 6 aborted foetuses and in one case (case n°9) of the mucosal disease affected animal was performed using Universal Alkaline Phosphatase Red detection kits (cat #760-501, Ventana Medical Solutions, Arizona, USA). Histology sections were stained with haematoxylin-eosin (HE) and examined under microscope.

### Table I: Sample description and results for BVDV detection

<table>
<thead>
<tr>
<th>Status / case</th>
<th>Age / sex</th>
<th>Year</th>
<th>Tissue</th>
<th>Histopathology</th>
<th>BVDV detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus / M</td>
<td>1991</td>
<td></td>
<td>Thymus</td>
<td>Lymphoid depletion</td>
<td>ND</td>
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<tr>
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<tr>
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<td>Liver</td>
<td>Autoysis</td>
<td>ND</td>
</tr>
<tr>
<td>Foetus / M</td>
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<td></td>
<td>Thymus</td>
<td>Lymphoid depletion</td>
<td>+</td>
</tr>
<tr>
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<td>Foetus / F</td>
<td>1993</td>
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<td>Thymus</td>
<td>No significant sign</td>
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<td>Foetus / F</td>
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<td>-</td>
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<tr>
<td>Foetus / F</td>
<td>1990</td>
<td></td>
<td>Thymus</td>
<td>Autoysis</td>
<td>-</td>
</tr>
</tbody>
</table>

### Buccal cavity

<table>
<thead>
<tr>
<th>Status / case</th>
<th>Age / sex</th>
<th>Year</th>
<th>Tissue</th>
<th>Histopathology</th>
<th>BVDV detection</th>
</tr>
</thead>
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<tr>
<td>7</td>
<td>6 month / F</td>
<td>2001</td>
<td>Oesophagus</td>
<td>of digestive mucosa</td>
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<tr>
<td>8</td>
<td>6 month / F</td>
<td>2007</td>
<td>Intestine</td>
<td></td>
<td>+</td>
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<tr>
<td>9</td>
<td>9 month / M</td>
<td>2009</td>
<td>Brain</td>
<td>Lymphoid depletion</td>
<td>+</td>
</tr>
</tbody>
</table>

M: male; F: female; *: Sex was not determined as the genitalia organs were scavenged; BVDV: Bovine viral diarrhoea virus; RT-PCR: Reverse transcriptase-polymerase chain reaction; FFPE: Formalin fixed paraffin embedded sections; FS: fresh sample; IHC: immunohistochemistry; FAT: Fluorescent antibody test; ND: not done.

TABLE I: Sample description and results for BVDV detection.
Detection of BVDV in archival tissue samples

Positive results appeared as bright red precipitate localized at the site of binding. Mounted tissue sections were barcoded and placed on the IHC automated staining system (Ventana Benchmark LT, Ventana Medical Systems, Inc., USA). Briefly, the slides were deparaffinised, treated with protease k2 at 37°C for 16 minutes for antigen retrieval then incubated with BVDV monoclonal antibody 15C5 (kindly donated by Syracuse Bioanalytical Inc., Ithaca, NY) at 1:1000 in antibody diluent (Dako, North America, Inc Ref: S0809) for 2 hours at 37°C. Coverslips were applied during incubation steps to avoid evaporation. Slides were washed and the primary antibody alkaline phosphatase labelled antibody complex were visualized using Fast Red Naphtol chromogen. Sections were counterstained with Haematoxylin for 4 minutes followed by 4 minutes in Bluing Reagent (Ventana cat #760-2037) then covered, dried and examined under microscope. Tissue from animal that was negative for both BVDV and antibodies was used as negative control while mucosal disease infected tissue was used as a positive control.

Molecular analyses

RNA extraction

RNA extraction from FFPE samples was performed using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion, Cat # AM1975) according to the manufacturer’s instructions. Briefly, sections were deparaffinised by adding 1 mL xylene and incubation at 50°C for 3 minutes then washed twice by 100% ethanol. Tissue sections were let air dried to remove residual ethanol. For RNA extraction samples were digested in Proteinase K digestion buffer while being incubated in a heat block for 15 minutes at 50°C, then 15 minutes at 80°C. Samples were treated with DNase and eluted with 60 µL elution solution then stored at -20°C. For RNA extraction from the reference virus BVDV1-NADL and BVDV2-125c strains, a commercial kit (Qiagen RNeasy Mini kit, Qiagen, Cat # 74106) was used. Total RNA was extracted from the supernatants of virus-infected cells following the kit instructions and RNA was eluted with 40 µL of RNase-free water and stored at -20°C.

RT-PCR screening

RT-PCR was performed using the primers (Table II) described by BAXI et al.[4] to amplify a 107 bp fragment. The assay was performed in a final volume of 20 µL containing 10.2 µL nuclease free water, 4 µL of 5X buffer, 1 µL 10mM dNTP mix, 1 µL of each PCR primer, 0.8 µL Enzyme mix and 2 µL extracted RNA. Prior to amplification, the RNA was transcribed at 50°C for 30 minutes. This was followed by one cycle of 94°C for 15 minutes for Taq polymerase activation and reverse transcriptase inactivation. This was followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 30 seconds and a final extension step at 72°C for 10 minutes. Positive control of BVDV NADL was used and master mix with elution solution instead of template was used as a negative control.

One step Multiplex Real time RT-PCR genotyping

Real-time multiplex RT-PCR was performed on all RT-PCR positive samples using Qiagen quantiTect Multiplex RT-PCR Kit (Cat # 204643). Real time RT-PCR was performed using the primers, probes (Table II), and protocol described by BAXI et al. [4] with some modifications, using Rotor-Gene 3000 (Corbett Research, Canada). A reaction of 25 µL volume containing 12.5 µL of QT master mix, 1 µL (400 nM) of each of forward and reverse primer, 0.5 µL (200 nM) of each of the fluorescent probe (FAM-BVDV1 and QUASAR- BVDV2), 0.25 µL of RT-mix, 4.25 µL of RNase free water and 5 µL of each of the RNA extracted from samples was prepared. Prior to amplification, the RNA was transcribed at 50°C for 30 minutes, followed by one cycle of 94°C for 15 minutes for the HotStar Taq DNA polymerase activation and reverse transcriptase inactivation. This was followed by 40 cycles of denaturation at 94°C for 15 seconds and of annealing and extension at 60°C for 60 seconds. Two positive controls of BVDV NADL and BVDV2- NVSL 125 were used and master mix with elution solution instead of template was used as a negative control.

Confirming amplicon sizes

Amplified PCR products were visualized in a 2% agarose gel in 1% Tris buffer EDTA and gels were stained with 1µg/mL ethidium bromide to confirm their sizes then photographed using UV light box and a camera.

Results

Pathology

As summarized in Table I, there was extensive autolysis in 6/16 samples from the 6 aborted foetuses which interfered

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers</td>
<td></td>
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<tr>
<td>Pesti-F</td>
<td>5'CTAGCCATGCCCCTTATAG3' 19</td>
</tr>
<tr>
<td>Pesti-R</td>
<td>5'CGTCGAACCAGTGACT3'</td>
</tr>
<tr>
<td>Probes</td>
<td></td>
</tr>
<tr>
<td>BVDV-1</td>
<td>5'-FAM-TAGCAACAGTGTGGTCTCCTGCCTG-3' 30</td>
</tr>
<tr>
<td>BVDV-2</td>
<td>5'-QUASAR-TAGCGGTTAGCAGTGGTCTGCAAGGTGGCC-BHQ-3'</td>
</tr>
</tbody>
</table>

Table II: Sequences of primers and probes used for BVDV detection.
with the histological interpretation of the sections. In few cases, depletion of the lymphoid follicles in thymus was observed. Lungs showed desquamation of the lining epithelium of the airways with peribronchial and interalveolar mononuclear cell infiltration. Meningeal blood vessels were congested in brain samples from 2 aborted foetuses.

In three cases (cases n°7, 8 and 9), characteristic lesions of mucosal disease were noted. The most striking lesions were small irregular erosions and ulcerations up to 1 cm in diameter on the tongue (figure 1), buccal mucosa and oesophagus (figure 3). Ulcerated areas were filled with fibrin, erythrocytes, necrotic debris and inflammatory cells and infiltration of the submucosa with large numbers of neutrophils and mononuclear leukocytes was also noticed (figure 3). Some areas of reddening with multiple petechiae on the Peyer’s patches were found in intestines. The colon showed linear areas of ulceration and the contents were black and pasty. The intestine showed severe congestion with erosions of the mucosa which were covered by fibrin, necrotic debris and some degenerated neutrophils.

### IMMUNOHISTOCHEMISTRY

In a mucosal disease affected calf (case n°9), intense positive BVDV immunostaining was evidenced in focal areas of eroded tongue mucosa and it often extended into the submucosa (figure 2). Strong BVDV antigen signals were also seen in the erosions of oesophagus (figure 4) and buccal mucosa (figure 5). In all examined tissues of the upper digestive tract, the highest intensity for the antigen signal was recorded in the stratum basale and stratum spinosum. The intestine showed positive staining for the BVDV antigen in the lamina propria and glandular epithelium of the mucosa, with scattered positively stained cells in the muscular layer (figure 6). A very weak antigen signal was obtained in the hepatic tissues particularly in the Kupffer’s cells whereas renal tissues showed moderate immunoreactivity in the glomerular tufts and interstitial tissues (figure 7).

Scattered positive signals were noticed in the interstitial tissues as well. The cerebral cortex showed strong antigen signal in the cytoplasm of the neurons and its processes and scattered glial cells showed immunoreactivity near the previously described neurons (figure 8). In the pulmonary tissues, pneumocytes and cells of the interalveolar septa showed positive BVDV staining. The peribronchial blood vessels were strongly positive while the bronchial epithelium remained unstained (figure 9). Although no immunoreactivity was found in cardiomyocytes, neighbouring blood vessels showed positive antigen signals in the tunica intima and tunica media (figure 10). In spleen and lymph nodes, positive BVDV labelling was recorded in both white and red pulps, as well as in few cells in the trabeculae (figure 11). The ear pinna showed strong antigen signal in the hair follicles but a weaker reactivity in the cartilage (figure 12). Blood vessels consistently stained positive immunolabelling in most tissues mainly localized in the smooth muscles (tunica media) and in the endothelial layer at a lesser extent.

From the 6 aborted foetuses, only one case (case n°6) showed multifocal areas of positive staining for BVDV antigen in the pulmonary tissue (figure 13) and weakly stained lymphoid follicles in thymus (figure 14).

### MOLECULAR FINDINGS

An amplicon size of 107 bp was obtained from one paraffin block of an aborted foetus (case n°6) and from all paraffin blocks from calves with mucosal disease (cases n°7, 8, and 9) after RT-PCR. The multiplex real time RT-PCR typed all extracted RNA as BVDV-1 (Table I).

### Discussion

Diagnosis of BVDV induced abortion is always a challenge. As the BVDV may be present in foetal tissues after induction
DETECTION OF BVDV IN ARCHIVAL TISSUE SAMPLES

**FIGURE 3:** Oesophagus (from a 9 month old calf, case n°9). Extensive erosion (arrows) in the mucosa with infiltration of predominately mononuclear leukocytes (arrowheads) into the underlying submucosa. Haematoxylin-eosin, bar: 200 μm.

**FIGURE 4:** Oesophagus (from a 9 month old calf, case n°9). Strong BVDV antigen signal in the remaining epithelium (arrows) of the focally eroded mucosa with moderate staining of the mononuclear leukocytes (arrowheads) infiltrating the underlying submucosa. Immunohistochemistry, bar: 200 μm.

**FIGURE 5:** Buccal mucosa (from a 9 month old calf, case n°9). Strong BVDV antigen signal in the stratum basale and stratum spinosum of the eroded area (arrows) with scattered positively stained leukocytes (arrowheads) in the submucosa. Immunohistochemistry, bar: 50 μm.

**FIGURE 6:** Intestine (from a 9 month old calf, case n°9). BVDV antigen present in the lamina propria and glandular epithelium (arrows) of the inflamed mucosa with scattered positively stained cells (arrowheads) in the submucosa. Immunohistochemistry, bar: 100 μm.

**FIGURE 7:** Kidney (from a 9 month old calf, case n°9). BVDV antigen is present in the glomerular tufts (arrows) with scattered positive signals in the interstitial tissues (arrowheads). Immunohistochemistry, bar: 50 μm.

**FIGURE 8:** Cerebral cortex (from a 9 month old calf, case n°9). Strong BVDV antigen signal is present in the cytoplasm and processes of neurons (arrows), and in some glial cells (arrowheads). Immunohistochemistry, bar: 50 μm.
FIGURE 9: Lung (from a 9 month old calf, case n°9). Strong antigen signals in the smooth muscles of the peri-bronchial blood vessel (arrows), pneumocytes (arrowheads) and cells within the interalveolar septa (asterisks). Immunohistochemistry, bar: 50 μm.

FIGURE 10: Heart (from a 9 month old calf, case n°9). Blood vessels showing positive staining in endothelium (arrows) and in smooth muscles of the tunica media (arrowheads). Immunohistochemistry, bar: 50 μm.

FIGURE 11: Spleen (from a 9 month old calf, case n°9). Strong BVDV signal is seen in the periphery of the white pulp (arrows) and in the red pulp (arrowheads) with sporadic positively stained cells in the trabeculae (asterisks). Immunohistochemistry, bar: 100 μm.

FIGURE 12: Ear pinna (from a 9 month old calf, case n°9). Strong BVDV immunostaining in multiple hair follicles (arrows), while the cartilage appeared weakly stained (arrowheads). Immunohistochemistry, bar: 100 μm.

FIGURE 13: Lung (from an aborted female foetus, case n°6). Strong BVDV antigen signal focally present in the pneumocytes (arrows) and cells within the interalveolar septa (arrowheads). Immunohistochemistry, bar: 50 μm.

FIGURE 14: Thymus (from an aborted female foetus, case n°6). Weak BVDV antigen staining (arrows) of the centrally depleted lymphoid follicle. Immunohistochemistry, bar: 50 μm.
of foetal persistency without abortion, thus virus isolation from tissues of an aborted foetus does not necessarily indicate that BVDV has caused such an abortion. Foetal antibody, on the other hand, indicates that foetal infection occurred in the later stages of gestation, and this is generally followed by virus clearance rather than an abortion [19].

The presence of only one BVDV positive case from the 6 examined aborted foetuses, in which BVDV was confirmed by FAT, may be attributed to the RNA–protein cross-linkage induced by the formaldehyde fixative [11]. Considering that samples were fixed in standard prepared fixatives, fragmentation of the nucleic acid that occurs with unbuffered fixatives can be excluded. Moreover, the quality of the extracted RNA is affected by many factors that include: type of the tissue, degree of autolysis, temperature and duration of the storage. All these factors can affect both the antigen retrieval for IHC staining and the quality of the extracted RNA for PCR amplifications [11]. On the other hand, cases 1-6 were selected as BVDV aborted foetuses based on FAT which was the screening test for BVDV during the early nineties. The accuracy of this test is questioned as it was associated with false negative and false positive results when compared to IHC [7].

There was a wide distribution of the BVDV antigens in the tissues of the mucosal disease affected calves. The BVDV demonstrated strong tropism for epithelial cells of the upper and lower digestive tract. The highest intensity of viral staining was seen in the eroded areas of the tongue, oesophagus and buccal mucosa particularly in the stratum basale and stratum spinosum. These results are in agreement with the previously described tissue antigen distribution of BVDV experimentally infected animals [8]. Our results are consistent with the previous study indicating the BVDV tropism to neurons and glial cells [15], as in our study there was strong antigen signal in the neurons and its process and also within some glial cells. There was moderate BVDV antigen signal in the pulmonary tissues despite the lack of significant lesions grossly. As previously reported in cattle with mucosal disease and in BVDV aborted foetuses, viral antigen was seen in the tunica media and tunica intima of blood vessels in lung, heart, kidneys and intestine [8].

In all the 9 investigated cases, there was a 100% agreement between the results of IHC and RT-PCR. All RT-PCR positive cases were genotyped as BVDV-1 by the one step multiplex real time RT-PCR. Similar results of presence of only BVDV-1 in the cattle of the Prince Edward Island region was obtained by AHMAD et al. in 2009 (unpublished data). The predominance of BVDV-1 in calves with respiratory disease was already described [10, 15]. Moreover, BVDV-1 was reported to be the most common isolate in the United States [17].

As a conclusion, BVDV can be detected efficiently by IHC or RT-PCR in formalized paraffin embedded tissues stored for up to two decades. Although, both IHC and RT-PCR are reliable tests, automation of the IHC and availability of the routinely processed FFPE tissue make it convenient for retrospective studies. The four BVDV positive cases in our study were typed as BVDV-1. This contributes to understanding of the epidemiology of BVDV infection in Prince Edward Island region, and improves the BVDV control program.

Acknowledgement

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References

1. - ALI H., ALI A.A., ATTA M.S., CEPICA A.: Common, emerging, vec-
tor-borne and infrequent abortogenic virus infections of cattle. Trans-
2. - ANDERSON M.L.: Infectious causes of bovine abortion during mid-
Assoc., 1987, 190, 1449-1458.
4. - BAXI M., MC RAE D., BAXI S., GREISER-WILKE I., VLCEK S.,
AMOAKO K., DEREGT D.: A one-step multiplex real-time RT-PCR for
detection and typing of bovine viral diarrhea viruses. Vet. Micro-
5. - BHUDEVI B., WEINSTOCK D.: Detection of bovine viral diarrhea virus
in formalin fixed paraffin embedded tissue sections by real time
6. - BROCK K.V., GROOMS D.L., GIVENS M.D.: Reproductive disease
and persistent infections. In: Bovine viral diarrhea virus: diagnosis,
management and control, Goyal S.M., Ridpath J.F. (eds), Blackwell
7. - ELLIS J.A., MARTIN K., NORMAN G.R., HAINES D.M.: Compa-
rison of detection methods for bovine viral diarrhea virus in bovine
436.
S., MARTIN K.M., HAINES D.M.: Lesions and distribution of viral
antigen following an experimental infection of young seronegative
calves with virulent bovine virus diarrhea virus-type II. Can. J. Vet.
Classification and Nomenclature of Viruses. Fifth Report of the Inter-
national Committee on Taxonomy of Viruses. Springer, New York.
10. - FULTON R.W., RIDPATH J.F., SALIKI J.T., BRIGGS R.E.,
CONFER A.W., BURGE L.J., PURDY C.W., LOAN R.W., DUFF
G.C., PAYTON M.E.: Bovine viral diarrhea virus (BVDV) 1b: pre-
dominant BVDV subtype in calves with respiratory disease. Can. J.
11. - GILBERT M.T., HASELKORN T., BUNCE M., SANCHEZ J.J.,
LUCAS S.B., JEWELL L.D., VAN MARCK E., WOROBEY M.: The
isolation of nucleic acids from fixed, paraffin-embedded tissues
12. - GROOMS D.L.: Reproductive consequences of infection with bovine
13. - HURTADO A., SANCHEZ I., BASTIDA F., MINGUIJÓN E.,
CONFE R A.W., BURGE L.J., PURDY C.W., LOAN R.W., DUFF
G.C., PAYTON M.E.: Bovine viral diarrhea virus (BVDV) 1b: pre-
dominant BVDV subtype in calves with respiratory disease. Can. J.
DONATO I., IOVANE G., GALIERO G.: Detection of Bovine viral
diabetes virus from three water buffalo fetuses (Bubalus bubalis)
15. - MONTGOMERY D.L.: Distribution and cellular heterogeneity of
bovine viral diarrhea virus antigen expression in the brain of persist-
tently infected calves: a new perspective. Vet. Pathol., 2007, 44, 643-
654.

