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XXI Cicle

"Evaluation of diagnostic techniques for the detection of Bovine Viral Diarrhoea Virus (BVDV) in infected and persistently infected cattle"

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ABSTRACT

Bovine Viral Diarrhoea Virus (BVDV) is a significant pathogen associated with gastrointestinal, respiratory, and reproductive diseases of cattle worldwide. The ability of BVDV to cross the placenta during early pregnancy can result in the birth of persistently infected (PI) calves, which are capable of shedding large quantities of virus throughout their lives and are considered the primary reservoirs for BVDV. PI animals do not show antibody response, and diagnostic tests such as virus isolation, immunohistochemistry (IHC), Antigen-Capture ELISA (ACE), and RT-PCR are used in their detection. Control efforts based on the identification and elimination of PI animals have been successful in a number of countries around the world. In Italy, little research has been done concerning BVDV prevalence and at this time no compulsory/voluntary control program exists. In order to investigate the seroprevalence and to identify PI animals in Central Italy, a serological survey on 1,929 unvaccinated Holstein Friesian animals from 21 farms from the Marche, Umbria and Lazio regions was carried out. Sera samples were tested to detect BVDV antibodies using a commercial ELISA kit (*Antibody BVDV IDEXX HerdCheck*). In order to detect PI cattle, all young (2 years old) and seronegative animals were tested individually using a commercial ACE (Serum/ACE BVDV IDEXX HerdCheck) and a RT-PCR protocol using pooled sera samples. In order to compare two different epidemiological situations, a short survey of 2 months was carried out at the Veterinary Diagnostic Laboratory of the Colorado State University (Rocky Ford, Colorado, USA) where a BVD voluntary program based on the identification and elimination of PI animals has been implemented. The protocol was carried out testing surnatants of ear-notches individually by ACE (ACE BVDV IDEXX, USA) derived from positive pools previously tested by RT-PCR. A total number of 14,508 samples were received, conforming 360 pools with an average number of 40 samples each one.

The results obtained in the serological survey of Central Italy demonstrated a total seroprevalence of 26.43%, meanwhile seroprevalences of 20.35%, 24.92% and 31.66% were observed in Umbria, Marche and Lazio regions respectively. These results showed the presence of BVDV infection with low prevalence. According to the age, 5.33% and 85.28% of animals younger and older than 2 years old respectively were positive. Only 10% (2/20) of farms showed recent infection of BVDV due to the presence of seropositive animals younger than 24 months old. All

seronegative / young animals tested (No. 490) were negative with the *Serum/ACE* using both, the standard and the overnight incubation methods with the intent to improve the sensitivity of the test, as well as by pooled RT-PCR. As additional *Serum/ACE* kit evaluation, 10-fold serial dilutions of BVDV TMV-2 strain in BVDV negative serum were tested, and positive results were seen in a 10^{-3} dilution confirming its validity. Therefore, no PI animals were identified in our research. Several factors such as the low PI prevalence, intermittence of BVDV excretion, the presence of a low percentage of PI animals that do not show detectable viral titers, along with the changing levels of viremia in PI animals must be considered. Our results demonstrate the presence of BVDV infection even in the absence of PI animals, illustrating the possible important role of transiently infected (TI) animals in the long term circulation of BVDV. Probably risk of indirect or external avenues of transmission must be considered due to the presence of on-going infections. In addition, the current herd management practices and low cattle density in Central Italy may be contributors to the low presence of PI animals. On the basis of these results the BVDV spread in Central Italy does not appear critical. The application of other diagnostic techniques such as IHC, RT-PCR and *ACE* using buffy coat and/or ear notches samples to detect the presence of PI animals is strongly recommended. In other hand and following the protocol of the Colorado State University, 15 Hereford of 4 months old animals were detected as PI animals, representing a total prevalence of 0.1%. This prevalence has been described everywhere, ranging from 0.5 to 2%. The methodology used offered a low-cost and highly sensitive system to detect BVDV infected animals.

Important differences as the number of animals sampled, the absence/ presence of a Control Program, the different samples used in each survey (serum vs. ear notch), differences in size, density and management, as well as the role of the presence of wild animals in the Colorado State are factors that must be considered between the different results observed in both surveys.

Keywords: Bovine Viral Diarrhoea Virus, Diagnostic Techniques, Persistent Infected Animals, Pooled Sera Samples, RT-PCR, Antigen Capture ELISA.

I. STATE OF THE ART

1. DEFINITION

The Bovine Viral Diarrhoea (BVD) is an infectious disease associated with pathology in several physiologic systems including reproductive, respiratory, gastrointestinal, circulatory, immunologic lymphatic, musculoskeletal, integumentary and central nervous (Brock KV., 2004a). BVD causes several sinology as pyrexia, diarrhoea, oculo-nasal discharge, depression, lymphopenia, thrombocytopenia, abortion, pneumonia and several secondary infections (Brock KV, 2004b). The virus has the ability to cause transplacental infection resulting in different outcomes depending on the stage of gestation at which the infection takes place, leading the presence of foetal death, malformation, acute syndromes of the neonate, immune tolerance and lifelong viral persistence (Peterhans E. *et al*., 2003). Because BVDV is an endemic disease in cattle populations in most parts of the world and due to its high prevalence and persistence, in combination with the negative effects on reproduction and the general health condition in dairy and beef herds, it is considered one of the most significant infectious pathogens in the livestock industry (Moennig V. *et al*., 2005a and 2006).

2. HISTORY

A disease of unknown origin affecting cattle was first described in the 1940s (Olafson P. *et al*. 1947). In western Canada, " X disease" was characterized by fever, watery and bloody diarrhoea, dehydration, tachypnea, anorexia, nasal discharge, hypersalivation and development of ulcers of the mucous membranes. Post-mortem findings were extensive, including erosive lesions in the respiratory and digestive tracts, together with a general enlargement of the lymph nodes. Later, in the Cornell University was reported an outbreak of an apparently new transmissible disease in cattle during the spring and summer of 1946 (Olafson P., *et al*. 1947; Brock VK., 2004a). The clinical signs and post-mortem findings were similar to those observed in " X disease" . However, Olafson observed that this new disease was reproducible, causing high mortality rates and it was associated with additional signs of respiratory disease, leucopoenia, drop in milk production and increased abortion rates. Finally in 1957, researchers isolated and cultured a virus from a similar case of infection accompanied with severe erosions and haemorrhages of the intestinal tract, which later was called mucosal disease (MD) (Underdahl NR,. *et al*., 1957). In this way, the unknown disease became Bovine Viral Diarrhoea (BVD).

By the end of the 1960, Scientifics speculated that MD and BVD disease were the same disease with minor variations. Then between 1960s and 1970s the research into the pathogenesis of the MD complex (BVD-MD) was focused on experimental infections, observing that abortions and teratogenic disorders were associated with intrauterine BVD virus (BVDV) infections. Such experiments also led to the understanding that neonatal calves congenitally infected with BVDV usually did not survive for more than a few months and eventually succumbing to what was previously described as chronic MD. These calves were discovered to be persistently infected (PI) with BVDV, and most importantly, PI calves were found to have an immunological abnormality because they did not produce detectable antibodies against BVDV (Brock KV., 2003).

At 1980s, BVDV was classified as a *Pestivirus*, together with border disease virus (BDV) and classical swine fever virus (CSFV). Cases of severe clinical disease, with severe gross and histological lesions caused by BVDV, were arbitrarily designated as MD. In contrast, the mild disease associated with minimal clinical changes from which BVD virus was isolated was called BVD. Until 1984, the researchers were able to reproduce classical MD with BVDV. Mucosal disease was replicated when a cytopathic BVD-MD virus was inoculated into a PI animal. It was subsequently established that MD occurs only in PI animals after super-infection with a strain of cytophatic BVDV that is antigenically similar to the non-cytophatic BVDV (Tautz N., *et al.,* 1998; Larson RL., 2006).

After the initial 30-year period, the characterization of BVDV as an RNA virus expected that mutation and variation would occur, and lately, the antigenic, genetic and pathogenic variation of the virus has been recognized. With the establishment of the major foundations (pathogenesis and biology) of BVDV, rapid advancements in the area of diagnostic detection including PCR, Real-Time PCR, immunohistochemistry and molecular epidemiology improve its research. Unfortunately, the adaptation to BVDV to cattle and the ability to cause persistent infections have given to the virus advantage to survive (Peterhans E. *et al*., 2003; Brock VK., 2004).

3. ETIOLOGY

BVDV is a member of the *Pestivirus* genus, belonging to the family *Flaviviridae,* which also includes border disease virus (BDV) and classical swine

fever virus (CSFV), three viruses that cause important financial losses to the livestock industry (Hornberg A., *et al*, 2008). *Pestiviruses* are small enveloped viruses of 40 to 60 *nm* diameter (**Fig. 1**). The genome consists of a single positive stranded RNA of about 12.5 kb (**Fig. 2**) (Tautz N*., et al*., 2003). The single open reading frame (ORF) is flanked by 5′ and 3′ untranslated regions (UTR). The 5' terminus of the genome is not capped, and the initiation of translation is mediated by an internal ribosomal entry site. The 5′ end of the genome, an UTR of about 370 bases, is immediately followed by a single ORF encoding for a unique polyprotein which is cleaved and matured into structural and non structural proteins of about 4000 amino acids (Murray CL., *et al*, 2007).

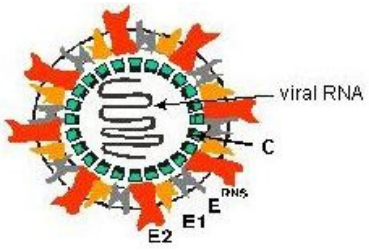


Fig. 1. *Pestivirus* structure

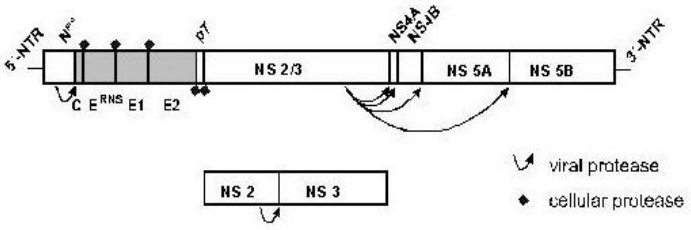


Fig.2. Genome organization and polyprotein processing of *pestivirus*

The polyprotein is processed by cellular and viral proteases into mature viral proteins, designated from the N terminus as structural (N^{pro}, C, E^{rns}, E1, E2, p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) **(Fig. 2)**. N^{pro}, the first viral protein translated in the genome, possesses an auto-proteolytic activity responsible for cleavage at its own C terminus. Next encoded in the polyprotein are the viral structural proteins, capsid (C) and three virion-associated glycoproteins, E^{rns}, E1, and E2. Several reports have shown that E2 is a major target of neutralizing antibodies, suggesting a significant role in receptor-mediated viral entry (Pankraz A., *et al*., 2005). E^{rns} has been reported to have intrinsic RNase activity, but its role in viral infection remains ambiguous. The BVDV genome encodes non-structural (NS) proteins, which are principally involved in viral replication (Sun JH., *et al*., 2003).

BVDV uses a strategy similar to that of other positive-strand RNA viruses to replicate (**Fig. 3**). Upon infection of cells, the genomic RNA serves as mRNA and is translated to produce the viral nonstructural proteins which are necessary for BVDV replication. Viral RNA replication is initiated by synthesizing a full-length minus-strand RNA complementary to the genomic plus-strand RNA. This minus strand then

serves as the preferred template for synthesis of additional plus-strand RNA molecules. Both minus and plus-strand viral RNAs can be detected at 4 h post-infection. Progeny virus can be detected as early as 8 h. *Flavivirus* RNA synthesis is localized to endoplasmic reticular membranes in the peri-nuclear site of infected cells (Lee YM., *et al*, 2005; Murray CL., *et al*, 2007).

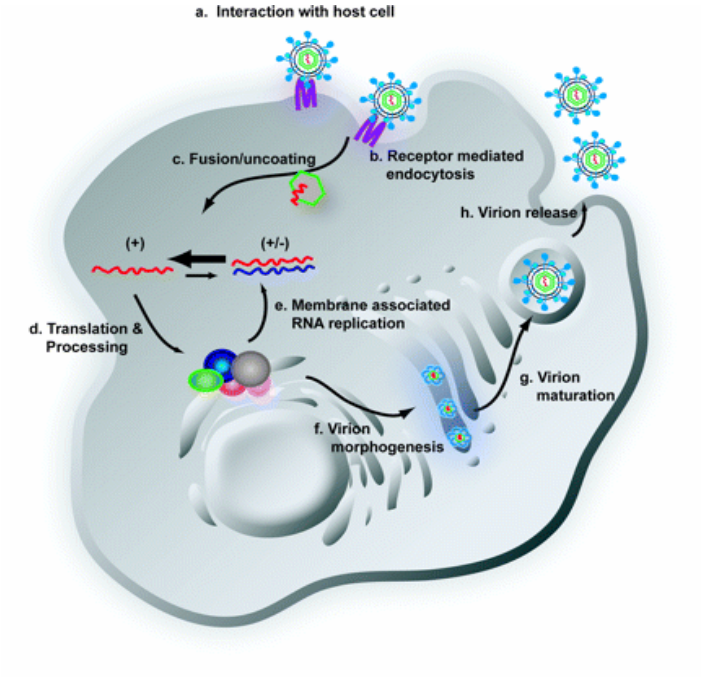


Fig. 3. General *Pestivirus* replication cycle

Mutations are frequent in RNA viruses and have been estimated as occurring about once per viral cycle for 10 kb, which is approximately the size of the BVDV genome. This means that any base in the viral genome is expected to undergo mutation once in every 10,000 replications of the viral RNA and it is attributable to the error-prone viral RNA polymerases responsible for replication of viral RNA (Bolin SR., *et al.*, 2004). Genetic recombinations may also arise when two different virions co-infect the same cell, allowing crossing-over between two genomes and resulting in a hybrid RNA (Desport M., *et al.*, 1998). Mutated viruses may be viable but disadvantaged as compared to the parent virus, but some mutations may result in the modification of viral antigens which will eventually allow to escape the host' s immune response (Bolin SR., *et al.*, 2004).

3.1. BVDV Genotypes and Biotypes

On the basis on their ability to induce microscopically visible changes (vacuolization and lysis) in host cells *in vitro*, BVDV isolates are divided into two biotypes (**Fig. 4**) (Birk AV., *et al*., 2008; Neill JD., *et al*., 2008). Cytopathic (CP) isolates cause changes in host cells, while noncytopathic (NCP) isolates do not cause cellular changes. Both biotypes of BVDV infect cattle and cause disease, but only the NCP biotype causes persistent infections (Kelling CL., 2004). The molecular basis of the CP effect is the synthesis of an additional protein of 80 kDa (called NS3 protein) produced in cells infected by a CP virus (Donis RO., *et al*., 1991). Biotypes apparently behave differently *in vivo*. NCP strains have a tropism for leucocytes, lymphoid organs and the respiratory tract, while CP strains are more or less restricted to the digestive tract. With the exception of MD cases, the majority of BVDV isolates from the fields are of the NCP biotype (Bezek DM., *et al*., 1994). CP strains would accidentally derive from this reservoir by mutation and would constitute an epidemiological dead-end. Lately, Ridpath (2006a) has suggested a third classification of a NCP BVDV strain, proposed as lymphocytopathic biotype which correlates with high virulence in acute infections in vivo. This biotype is able to shows only visible change in cultured lymphoid cells, concluding that belongs to a low virulence NCP biotype.

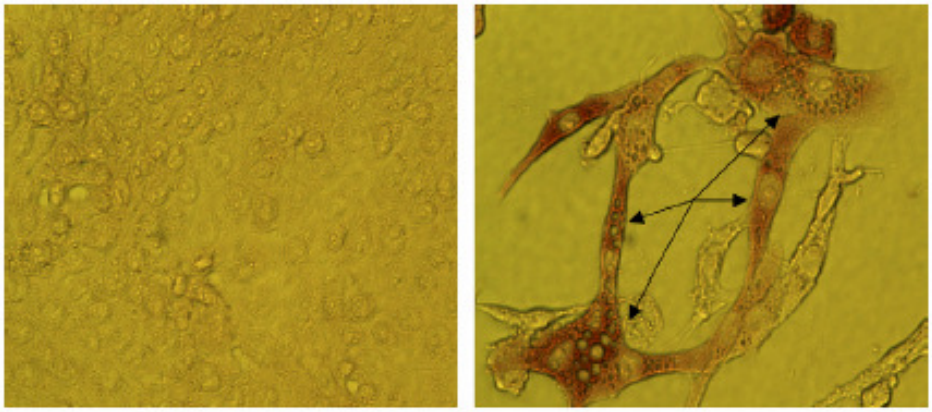


Fig. 4. Infected MDBK cells with NCP (left) and CP (right) BVDV. Arrows indicate the cytoplasmic vacuolization induced by BVDV CP strains.

On the basis of the nucleotide sequence of the 5' UTR, two genotypes of BVDV have been described (Ridpath JF., 2003; Vilcek S., *et al*., 2005). Genotype 1 includes the classic BVDV isolates, which are commonly used in laboratory

reference and vaccine strains. Type 1 is subdivided into two types 1a and 1b, but a recent study indicated that BVDV could be clustered into 11 phylogenetic groups (Vilcek S., *et al*., 2001). In other hand, genotype 2 comprises the BVDV strains associated with high mortality acute and per-acute infections, thrombocytopenia and haemorrhaging. Although both genotypes cause disease, the clinically severe, acute BVDV infections are caused by NCP type 2 BVDV (Giangaspero M., *et al*., 2008).

In cattle populations BVDV-1 strains are predominant in most part of the world, whereas BVDV-2 is recognized as the cause of severe acute haemorragic disease in North America (Pellerin C., *et al*., 1994), being recently reported in Europe and Asia with low virulence (Letellier C., *et al*., 1999; Luzzago C., *et al*., 2001). In Italy, BVDV-1 is the predominant and most wide distributed genotype in the north of the country, where 30% of the bovine population is concentred. The BVDV-1b is the most frequently isolated, followed by groups c, d and e (Luzzago C. *et al*., 2001; Falcone E. *et al*., 2001 and 2003). Recently, Giammarioli M. *et al.,* (2008) have reported that from 88 BVD viruses found throughout Italy, genetic studies based on the 5'-UTR showed the presence of subgenotypes BVDV-1a, 1b, 1d, 1e, 1f , 1g and 1h. In case of BVDV-2 genotype, it has been isolated from 2 healthy animals from a farm in the northern Italy where a several outbreak of BVDV was present few months before the screening (Luzzago C. *et al*., 2001), and in a several outbreak originated for the use of Herpesvirus-1 Modified Lived Vaccine (MLV) contaminated with BVDV-2 strain (Falcone E. *et al*., 1999). The presence of BVDV-2a has also been demonstrated in 5 of 88 BVD viruses found throughout Italy (Giammarioli M., *et al.*, 2008).

4. EPIDEMIOLOGY

Transmission of BVDV occurs both horizontally and vertically (**Fig. 5**) (Lindberg A., *et al*., 2005 and 2006). In an infected herd, there are two principally different sources of virus: PI animals and animals that undergo a transient infection (TI) (**Fig. 5**). Direct contact between an infected or PI animal and susceptible animal is considered the principal route of transmission. The horizontal transmission of BVDV may be direct or indirect via inhalation or ingestion of virus contaminated materials. Horizontal transmission occurs mainly by contacts with virus-shedding, but PI and TI animals excrete the virus in different amounts (Houe H., 1995).

The probability of transmitting BVDV by other means than nose-to-nose contact is dependent on the dose and on the means of contact. The virus persists in the environment for more than 2 weeks and can be excreted by saliva, ocular or nasal secretions, urine, semen, uterine secretions, amniotic fluid, placenta and vaginal mucus. Infected bulls can shed BVDV in semen for prolonged periods, and cattle have been infected following insemination with frozen semen from these animals (Houe H., 1995; Gard JA., *et al.*, 2007). Experimental studies have shown that various equipment as nose tongs and rubber stopper on a vaccine vial contaminated serve as a vector for infection (Fray MD., *et al.*, 1998; Lindberg A., *et al.*, 2006).

In other hand, BVDV passes vertically by transplacental infection from infected dams to the foetus. BVDV uses the productive system to maintain and spread itself in the cattle population by inducing immunotolerance following foetal infection, resulting in birth of PI calves that are considered the most important source of infection (Loneragan GH., *et al.*, 2005).

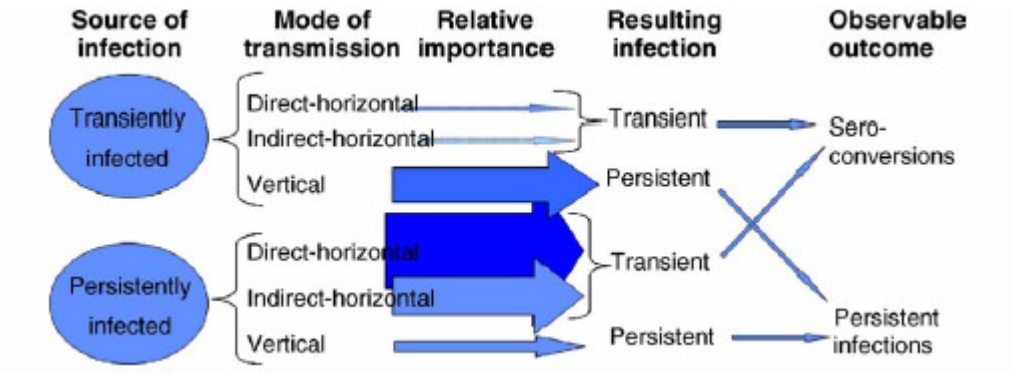


Fig. 5. Relative role of TI vs. PI animals as source of virus for horizontal and vertical transmission of BVDV and the possible outcomes within an infected herd (Lindberg A., *et al.*, 2005).

4.1. Persistently Infected animals (PI)

The definition of BVDV PI animal is one that BVD virus has been isolated from peripheral blood leukocytes or serum in two separate occasions at least 3 weeks apart (Brodersen BW., 2004). The prevalence of BVDV PI cattle has typically been observed in the range of 0.5% to 2% (Brock KV., 2003; Peterhans E., *et al.*, 2003; Smith DR., *et al.*, 2004), although the prevalence of PI within herds is variable.

The age-specific prevalence of PI is greatest at birth and decreases with age. Approximately 50% of BVDV PI cattle will die during the first year of life (Smirnova NP., *et al.,* 2008).

PI animals play a substantially larger role in transmitting the virus than TI animals do. They shed virus in high concentrations in all bodily fluids during their entire life (Brock KV., 2003; Muñoz-Zanzi CA., *et al*., 2000 and 2003). The blood of PI animals can contain up to 10^7 $TCID_{50}$/mL BVDV (Perry GH., 2007). However, empirical evidence supports the importance of PI animals for within-herd spread in that the infection essentially stops when PI animals are removed, which makes it possible to clear infected herds from the virus without taking special measures (Lindberg A., *et al.,* 2005).

The effect of the massive doses of virus shed by PI animals on within-herd spread of BVDV is evident from studies showing that airborne transmission can occur within buildings, and tools as equipment and pens where PI animals have been housed can (for a limited period of time) carry a sufficient dose of live virus for susceptible animals to seroconvert (Niskanen R. and Lindberg A., 2003). In comparison to TI cattle, horizontal transmission of virus from PI animals to susceptible cattle is more efficient.

Some reports have shown that after PI animals are born, more than 90% of cattle in the herd before 3±4 months of age get infected. This was also seen in the study by Moerman A. *et al*. (1994), where all susceptible cattle that came into contact with a PI animal became seropositive within 3 months. In another study, seronegative animals seroconverted within 5 months after the introduction of a PI animal and at short distances, the animals had seroconverted within 2 months, whereas at longer distances the time of seroconversion was more undefined.

In beef herds, suckling calves are commonly in contact with the breeding herd during early gestation when the foetus is susceptible to BVDV persistent infection before 125 days of gestation. As a result, PI suckling calves are considered the primary source of BVDV infection in breeding herds, causing decreased conception rates, loss of pregnancy, pre-weaning mortality, and induction of PI calves in the next year' s calf crop (Ezanno P., *et al,* 2008).

Cattle transiently infected (TI)

TI animals play a less significant role in persistence and spread in BVDV herds, but do affect the general health of herd. TI cattle can be the primary source for introduction of BVDV and initiation of PI, but they are inefficient sources of horizontal virus transmission (Brodersen BW., 2004; Smith DR. *et al.*, 2004). The probability of horizontal transmission during a contact with a TI animal stands in sharp contrast to that of PI animals, due to the intermittent shed of relatively low amounts of virus (Niskanen R, Lindberg A., 2003) . There are experimental results that indicate that the primary infection may be only rarely propagated and TI animals, unless they are in early pregnancy, are the end for the infection (Lindberg A. and Houe H., 2005). The probability of vertical spread resulting from a TI is much higher than the probability of horizontal spread. In fact, most new PI detected in an infected herd will be the result of TI in dams with a normal immune response. However, there are some reports that suggest that TI can circulate for long periods of time (10 months or more) even in the absence of identified PI animals (Moerman A., *et al.*, 1993). In two studies, the virus circulated for 2±2.5 years although there were no PI animals present and no direct contact with PI cattle was established (Moen A. *et al.*, 2005).

The length of the contact can be permanent, meaning that the infectious animal is permanently introduced into the herd, or transient, where the infectious animal is in contact with other herds for a limited period of time, (e.g. at a livestock market, a common pasture, a transportation, etc.). The probability of transmission will depend on whether the infectious animal is in contact with a pregnant recipient or not, as well as the duration of their infectious period (Lindberg A. and Houe H., 2005).

4.3. The role of biologicals in BVDV transmission

The biologicals (vaccines, cryopreserved embryos and semen) as vehicles for BVDV infection are potential to transmit the virus not only to neighbouring herds or between regions or countries, but also between continents (Stringfellow DA., *et al.*, 2005). As vaccines or semen from the same batch/bull will be used in many herds, the extent of such transmission can be vast and the consequences severe (Givens MD., *et al.*, 2003).

Viral contamination of semen can result from four distinct types of infections with BVDV: a) PI bulls, b) acute infection of bulls due to exposure after development of immune-competence and c) prolonged testicular infection of bulls due to an enduring infection of testicular tissue after acute infection (Gard JA., 2007).

PI bulls could shed large quantities of BVDV in semen ($10^{7.6}$ $CCID_{50}$/mL) which can survive processing and cryopreservation. This contaminating virus will consistently infect susceptible, inseminated heifers and cows and occasionally may result in production of PI calves. Acutely infected bulls can shed BVDV in semen from 2 to 20 days after infection. The concentration of BVDV in these contaminated semen samples (5– 75 $CCID_{50}$/mL) is much less than detected in semen of PI bulls (Gard JA., 2007; Rikula U., *et al.*, 2008). Resulting acute infections can cause proliferation of BVDV within a herd which results in new PI developing foetuses, creating a temporal window for transmission of small quantities of virus on a limited basis to susceptible semen recipients. Thus, semen from PI or acute infected bulls creates a risk of viral transmission via artificial insemination, in vivo embryo production, and in vitro embryo production (Bielanski A., *et al.,* 2008).

In the same way, the presence of potentially live virus in foetal-calf sera is also suggested as probable way in which BVDV could be transmitted by embryo transfer. Such products are regularly used in the production of in vitro fertilised embryos and also for in vivo produced embryos. Embryo processing (i.e. washing or trypsin treatment) will certainly reduce the level of contamination should embryos be exposed to the virus (Lindberg A. and Houe H., 2005).

In other hand, contamination of Modified Lived Vaccines (MLV) with adventitious viruses, particularly virulent BVDV, during the manufacturing process has represented a significant risk factor since these products were introduced in the world marketing. The main source of adventitious BVDV is foetal bovine serum used as supplement in cell culture medium used in cell cultures to grow vaccine virus. Between 20% and 50% of commercial foetal bovine serum have been identified positive for both genotypes, predominating genotype 1 of BVDV (Houe H., 1995). In recent years, confidence in purity of MLV BVDV vaccines has increased, as more attention has been focused on this issue and as improved test have been used as RT-PCR and others techniques. Foetal bovine serum that pass rigorous testing procedures and are shown to be negative for virus may be irradiated or

chemically treated as an additional precautionary measure before being used in cell culture production systems to grow vaccine virus (Kelling CL., 2004).

4.4. The role of non bovine host

Infected cattle is considered the most possible source of infection for other herds. If there are other sources of the infection that cannot readily be controlled by bio-security measures, the prospects for successful BVDV control could be hampered. In fact, the presence of non-bovine hosts of BVDV has been put forward as a reason why eradication could not be achieved in some situations (Houe H., 1995).

As suggested in previous reports, areas where wild ungulates and cattle breed and graze synchronically have theoretical precondition for transmission (Anderson EC., *et al*., 1998). However, in order for wildlife to act as a long-term reservoir, the virus needs to be able to infect cattle and persist in wildlife populations without being reintroduced (Vilcek S., *et al*., 2000).

Pestiviruses are not strictly host-species specific and can infect not only domestic but also wild animals. Serological examinations provide evidence for the contact of free-living animals as cervidae, roe deer, red deer, fallow deer, mule deer, white-tailed deer and caribou. BVDV antibodies were also found in buffalo, giraffe, bighorn sheep, eland, chamois, pronghorn antelope, camelids and many other wild animals mostly from Africa and America. Different researches have shown that BVDV can replicate in species such as camels, deer, elk and bison. Natural infection has been described also in pigs, alpacas, sheep, goats, buffalo and wild deer (Nielsen SS., et al., 2000; Frölich K., *et al*., 2006; Duncan C., *et al*., 2008).

Currently and due to the prevalence of BVDV, it is difficult to measure or predict the importance of secondary hosts in the maintenance of the virus in cattle populations. As the prevalence of BVDV is reduced by prevention and control methods, it is likely that the significance of infections in secondary hosts will be comprehended (Brock KV., 2003).

4.5. Risk factors

Epidemiological investigations have shown that demographic factors such as herd size and density are significant predictors for the prevalence of infection in populations where BVDV is endemic (Houe H.,1995; Valle PS.,*et al*., 1999).

Epidemiological data show that BVDV spreads more efficiently in areas with high cattle density. In such areas, where more herds will tend to be BVDV positive, the economical losses are often moderate and constant. Conversely, in areas whit low cattle density, more herds are likely to remain free from BVDV even without active control measures (Ezanno P., *et al.,* 2008; Talafha AQ., *et al*., 2008). If BVDV is introduced to naive breeding herds, initial losses due to reproductive failure or clinical disease in calves infected in uterus may be substantial, but once adult animals have gained immunity to the virus, the continued BVD-related losses will be lower (Sandvik T., 2005). A significant higher seroprevalence and infection level have been observed in medium and large size herds than small herds. As well, higher seroprevalences are observed in herds that purchase animals from different sources. Thus, important risk factors as herd size, exchange of visits between adjacent farm workers, absence of calving pens, contact with cattle from other herds such as pasturing at close distance, fence break out, animal shows and the lack of isolation of purchased animals, have an important influence on spread and transmission of BVDV (Lindberg A. and Houe H., 2005; Ezanno P., *et al.,* 2008).

5. PATHOGENESIS OF BVDV INFECTIONS

5.1. Acute infections

Acute infection of immunocompetent cattle with BVDV can result in a wide range of clinical syndromes. The majority of acute BVDV infections are caused by NCP viruses. PI and TI animals are the primary source of virus, shedding virus mainly in nasal and oral secretions (Kelling CL.,2004). The primary virus entrance is probably by oral and nasal routes. Following entry and after the contact with the mucosal lining of the mouth or nose, viral replication occurs in epithelial cells with a predilection for the palatine tonsils, lymphoid tissues, and epithelium of the oropharynx. Phagocytes take up BVDV or virus-infected cells for transport them to peripheral lymphoid tissues, but the virus is also able to spread systemically through the blood stream. Viremia is evident 2 to 4 days after exposure and virus isolation from serum or leucocytes is generally possible between 3 and 10 days post infection (Fray MD., *et al*., 1998; Brackenbury LS., *et al*., 2003). During systemic spread, the virus is able to gain entry to most tissues with a preference for lymphoid tissues. However, the tissues infected may vary between different virus strains. Following acute infection, it is generally accepted that most clinical outcomes are mild as fever,

diarrhoea, and leucopoenia (Booker CW., *et al.,* 2008). However, some CP strains have been associated with much more severe disease including fatal hemorrhagic diarrhoea and thrombocytopenia (Bezek DM., *et al.,* 1994). Secondary infection by other pathogens may occur because of immunossupression. Finally, antibodies effectively neutralize viral infectivity, promote clearance of virus, and prevent seeding of target organs and foetus (Chase CC., *et al*., 2004; Smirnova NP., *et al*., 2007).

5.2. Intrauterine infections

Throughout acute infections, infected cows could course a non-suppurative oophoritis with necrosis of granulosa cells, reducing the fertility rates consecutively (Grooms DL., 2004; Larson RL., 2004 and 2006). During pregnancy, BVDV has the ability to cross the placenta and cause intrauterine infections. The outcome of these infections are largely dependent on the stage of gestation when infection occurs. Embryos appear to susceptible following implantation which occurs at day 20 of gestation. During this early phase of pregnancy, infection with BVDV often results in early embryonic death and resorption. Foetal infection during the first trimester of gestation can result in abortion and foetal mummification. If the foetus survives to this early infection, they invariably become PI and immunocompetent (Brock KV., 2003; Brodersen BW., 2004).

Foetal BVDV infection during the late first, second and early third trimester of gestation may also result in the formation of several different types of congenital anomalies. As the immune system develops during the second trimester of gestation, the foetus is able to mount an immune response to a BVDV infection, which results in the birth of clinically normal calves with BVDV pre-colostral antibodies (Browline J., 1998).

5.3. Mucosal disease (MD)

MD occurs only in cattle that had been infected with a NCP BVDV during the first trimester of gestation (PI animals) (Tautz N., *et al*., 1998), and occurs when these animals are superinfected by an antigenically homologous CP strain. Both viral biotypes, NCP and CP BVDV, are consistently found in animals that come down with MD (Kümmerer BM., *et al*., 2000; Bolin SR., *et al*., 2004). The super-infection of the PI animal with an antigenically similar CP BVDV can occur in several

ways. The most common occurrence is for a mutation of specific sites in the NCP virus genome. This mutation may occur in several different ways including insertion of RNA into or deletion of RNA from the NCP BVDV genome. The mutation does not change the antigenic make-up of the virus, therefore the CP virus is not recognized by the host's immune system and is allowed to replicate without challenge. Other sources of CP viruses would include MLV or experimental challenge. Antigenic homology between the CP and NCP virus must be maintained for MD to occur. Replicating CP BVDV results in rapid depletion of the gut-associated lymphoid tissue (Peyer' s patches) with subsequent necrosis of the gastrointestinal mucosa (Hamers C., *et al.*, 2001).

6. CLINICAL SIGNS

There is some evidence that a particular type of BVDV correlates with a defined clinical-pathologic presentation (**Fig. 6**). The practical significance of biotype is that, in vivo, NCP viruses may establish persistent infections but CP viruses do not. NCP viruses predominate in nature, meanwhile CP viruses are relatively rare and usually found in association with outbreaks of MD, a relatively infrequent but highly fatal form of BVDV infection (Houe H., 1995 and 2003; Lindberg A., *et al.*, 2005). Cytopathology in vitro does not correlate with virulence in vivo (Bezek DM., *et al.*, 1994) and the most clinically severe form of acute BVDV infection is associated with NCP virus (Pellerin E., *et al.*, 1994; Ridpath JF., *et al.*,1994). NCP BVDV-1 strains are more correlated with respiratory disease than CP BVDV-2 genotypes (Baule C., *et al.*, 2001). Clinically severe BVDV, also known as severe acute BVDV (SA-BVDV), occurs in animals infected with a type 2 BVDV that have no or low titers against BVDV2 strains. It is associated with a greater than 50% reduction in circulating lymphocytes and platelets and body temperatures exceeding 40.6°C. Severe acute BVD may progress to hemorrhagic syndrome in some cases (Stoffregen B., *et al.,* 2000; Liebler-Tenorio EM. *et al.*, 2003).

6.1. Subclinical BVD Infections

Both NCP and CP-BVD biotypes are associated with subclinical BVD infections. Approximately 70-95% of the animals that become infected with the BVD virus do not develop signs of disease that are directly caused by the virus. However, subclinical infections may be accompanied by a decrease in meat or milk production

and a reduction in reproduction performances (Moerman A., *et al.*, 1994; Dubovi EJ., 1994). Generally, BVD subclinical infection causes a decrease in the animal's resistance to other secondary infections. These infections quite often are not recognized as being initiated by the BVD virus. For example, when the BVD virus infects the lungs of calves, the virus causes little or no disease. However, the virus interferes with the ability of the lungs to rid themselves of bacteria that are found routinely in the respiratory tract. The BVD virus has been identified as one of the most significant disease organisms involved with respiratory disease of cattle (Baule C., *et al.*, 2001).

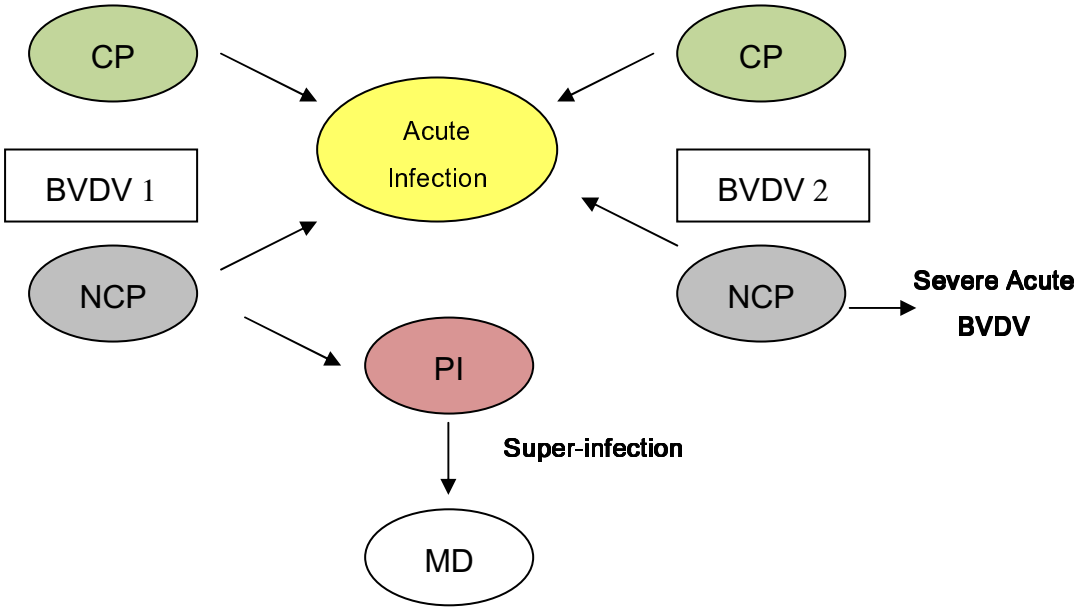


Fig. 6. Principal outcomes after BVDV infection with differente genotypes and biotypes

6.2. Peracute BVD Disease

This disease syndrome is usually associated with the Type 2 NCP BVDV infection. The affected animals will exhibit high fever, occasional diarrhea, respiratory disease, and loss of appetite or anorexia. Peracute BVD can affect cattle of all ages and often results in the death of the animal within 48 hours from the onset of disease (Kelling CL., 2004).

6.3. Acute BVD Disease

The classic acute BVD form can be caused by either CP or NCP strains, but are most common caused by NCP strains. The infection may result in a wide range

of clinical manifestations, varying from clinically unapparent to severe. BVDV replicates in monocytes, B cells, T cells, and epithelial cells of the gastrointestinal and upper respiratory tracts; and in this way the virus contributes to cause respiratory disease and enteritis (Baule C., *et al*., 2001). The infection is characterized by high morbidity, low mortality, normal host immune response and minimal mucosal lesions. Signs as fever, discharge from the nose and eyes, erosions of the muzzle and in the mouth, severe and transient hemorrhagic watery diarrhoea, rapid respiration, ptyalism, inappetance, coughing, depression, lymphopenia and trombocytopenia, petechial and ecchymotic hemorrhages, bleeding from injection sites, abortion and pneumonia could be observed (Odeon AC., *et al*., 2003; Kelling CL, 2004; Smith DR., *et al*., 2004; Dabak M., *et al*., 2007).

The clinical picture can vary from animal to animal, especially as it relates to the presence of erosions and diarrhea. Diarrhea is usually present in every herd that has an outbreak of acute BVD, but diarrhea is not present in every animal that has acute BVD. In susceptible herds, clinical signs usually affect animals ranging from 6 to 12 months old (Baker JC., 1995). The virus can be recovered from blood and nasal secretion of acutely infected cattle from 6 to 8 days, and antibodies titers rise slow for 3 months post-infection. The slow increase of antibody titter may be due to the immunosuppressive effect of the virus (Peterhans E., *et al*., 2003). Acute BVDV infections play an important role as immunosuppresive agent or as potentiator of other diseases. Although the majority of acute infections are subclinical, acute infections are also occasionally identified as primary cause of mortality in calves. The profound immunosupresive effect of acute BVDV are responsible for the potentiation of a variety of diseases in cattle, including bacterium diseases like *Salmonellosis*, *Escherichia coli, Mannheimia haemolytica, Mycoplasma bovis, Mycobacterium bovis*, and viral infections as rotavirus, coronavirus, IBR, etc. Acute BVD infections in the newborn calf may be more prevalent than is currently recognized because the disease is usually masked by secondary infections that cause diarrhea and/or pneumonia (Potgieter LN., 1995; Campbell J.R., 2004).

6.4. Chronic BVD disease

The chronic form of BVD is associated with prolonged BVD virus infections and very poor or absent antibody titer to the BVD virus. The clinical signs associated with the chronic disease are more sever than the clinical signs associated with the

acute BVD disease. The chronically diseased animals exhibit depression, a lack of appetite, a lingering diarrhea, a yellowish discharge from the eyes and nose, crusted muzzle, erosions of the mouth, bald spots due to loss of hair, and lameness due to inflammation of the hair line, sensitive laminae, and the tissue between the claws of the feet. The chronically infected animals usually appear unthrifty and starving. Death occurs more frequently in chronic BVD infections than in acute BVD infections (Odeon AC., *et al*., 2003; Liebler-Tenorio EM., *et al*., 2003).

6.5 Reproductive signs

BVDV can cause a wide array of reproductive losses that are largely dependent on the time of gestation when infection occurs (**Fig. 7**).

- **Infection from 0 to 45 days of gestation**. BVDV reduce significantly the conception and pregnancy rates in infected animals, maybe because it is associated with the failure of fertilization or early embryonic death (EED). Several studies have shown that following an acute infection with CP BVDV, the animals develop an interstitial oophoritis with lesions lasting up to 60 days. Other features as infection and subsequent viremia during preovulatory phase, hypoplastic ovaries, infertility, salpingitis for up to 21 days and the decrease of viability of blastocyst have been described (Smith DR., *et al*., 2004; Grooms DL., 2004).
- **Infection from 45 to 175 days of gestation**. Following implantation, transplacental infection of the developing foetus can occur in susceptible cows with either biotype of BVDV. The outcome of the infection is largely dependent on the timing of the infection:

a) Abortion. Under experimental conditions, both CP and NCP BVDV viruses can cause foetal death following infection of seronegative dams. This can occur at any point during gestation, although it is most common during the first trimester. Foetal resorption, mummification or expulsion can also occur depending on the time of infection (Swasdipan S., *et al*., 2002; Greiser-Wilke I., *et al*., 2003).

b) Immunotolerance. Non-lytic infections produced by NCP BVDV strains and the ability to evade the host immune response are the primary mechanisms of persistence (Brock KV., 2003). Foetus that survive infection with NCP BVDV between 18 and 125 days of gestation invariably develop immunotolerance to the

virus and subsequently become PI with BVDV. This mechanism is due because the virus proteins are recognized as self-antigens, resulting in a negative selection of BVDV specific B and T lymphocytes. NCP BVDV is the only biotype that has been able to natural and experimentally produce persistence (Greiser-Wilke I., *et al*., 2003; Ridpath JF., 2003 and 2006a).

c) Congenital defects. Foetal infection between 100 and 150 days of gestation, often results in the development of a variety of congenital defects. During this stage of gestation, organogenesis is being completed and the immune system is becoming fully functional. The NCP biotype can replicate in the early foetus, causing damage to selected tissues but not sufficient to cause death. Congenital anomalies as hypomyelination of the central nervous system, microencephalopathy, hydrocephalus, hydranencephaly, porencephaly and cerebellar hypoplasia are observed. The cerebellar hypoplasia is the most common and the calves born with this disorder show tremors, ataxia, wide-based stance and stumbling gait. Other teratogenic effects associated to BVDV are cataracts, microopthalmia, retinal degeneration, optic neuritis, hypotrichosis, alopecia, mandibular brachygnathism and growth retardation (Riond JL., *et al*., 1990; Grooms DL., 2004; Smith DR., *et al*., 2004).

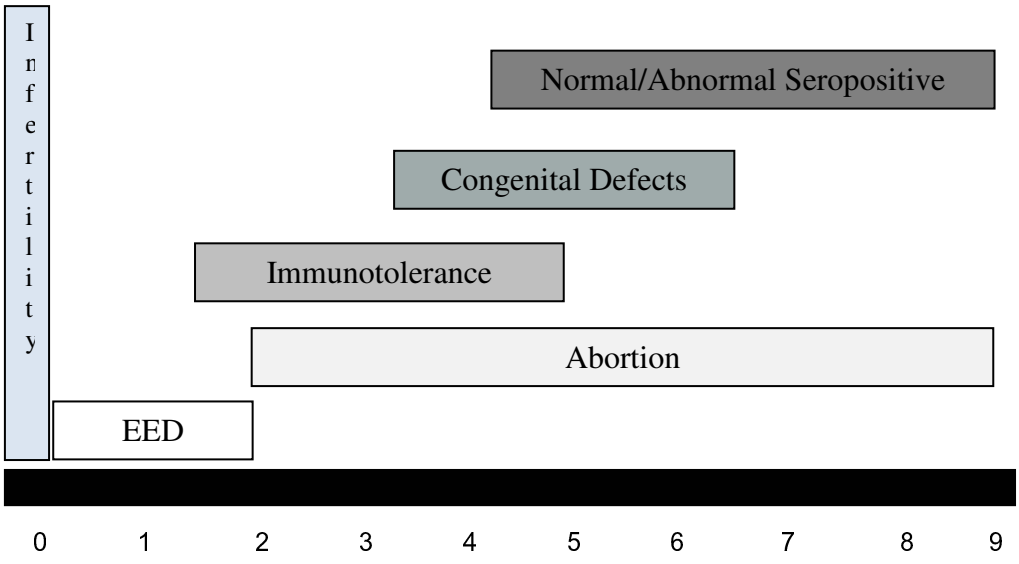


Fig. 7. Potential clinical reproductive outcomes following infection with BVDV. EED: early embryonic dead (Grooms DL., 2004).

- **Infection from 125 to 285 days of gestation**. In the later stage of gestation, immunocompetence and organogenesis are usually complete. Although abortions and the birth of weak calves have been attributed to infection with BVDV late in gestation, foetuses infected during this time period are normally able to mount an affective immune response and effectively clear the virus, having pre-colostral neutralizing antibodies to BVDV. Even that, some abortions and weak calves have been reported from BVDV infection during this period (Wittum TE., *et al*., 2001; Smith DR., *et al*., 2004).

6.6. PI animals

Calves PI are persistently viremic and shed the virus continuously (Houe H., 1999; Kuhne S., *et al*., 2005). PI calves may appear normal, but are frequently poor-doers having reduced growth rates, immunosuppression, increased morbidity and mortality because they are more susceptible to many calfhood disease, such as pneumonia. PI animals may also develop MD, usually before they are 2 years old (Odeon AC., *et al*., 2003). Erosions of the nose, muzzle and interdigital cleft and crustaceous dermatitis occasionally described in persistently BVDV-infected cattle and corroborate the potential of BVDV for tissue damage in such locations (Dabak M., *et al*., 2007).

6.7. Mucosal Disease (MD)

MD is a sporadic disease in cattle that often occurs between the ages of 6 months and 2 years. The disease is characterised by high case fatality with death occurring usually within 2 weeks after the onset of clinical signs (Baker JC., 1987). Extensive ulceration of the gastrointestinal tract is the most prominent lesion. Characteristic clinical signs of mucosal disease include anorexia, fever, diarrhea, dehydration, presence of lesions in the mucous of the digestive tract, necrosis of lymphoid tissue, hoof inflammation, and loss of condition and death (Wilhelmsen CL., *et al*., 1991;Kelling CL., 2004). Dermatitis is a sign frequently present in MD and is the common finding of the BVDV in skin biopsy specimens in PI cattle, confirming the tropism of the virus for the epithelia (Wilhelmsen CL., *et al*., 1991;Dabak M., *et al*., 2007).

7. IMMUNE RESPONSE

7.1. Innate response

INTERFERON. Interferon is the most important innate defence antiviral cytokine. Viral infections commonly result in the induction, release, and remote action of type I IFN' s, notably IFN-α, IFN-γ, as well as the newly described IFN-τ (Brackenbury LS., *et al*., 2003). Released IFN acts through specific receptors present on nucleated cells to transduce signals for the transcription of numerous genes, such as the genes encoding the double-stranded RNA (dsRNA)-dependent protein kinase (PKR), the 2' -5' -oligoadenylate synthetase (2' -5' -OAS). PKR and 2' -5' -OAS are produced as inactive precursor molecules. Once activated by viral dsRNA intermediates, these effectors act to inhibit the host' s cell transcription machinery and ultimately contribute to the induction of apoptosis of virus-infected cells. However, NCP virus inhibits double-stranded RNA that induces apoptosis and the interferon synthesis. This phenomena is associated with the establishment of persistence in the bovine fetus (Peterhans E., *et a.l,* 2003; Chase CC*., et al,* 2004).

APOPTOSIS. This mechanisms may be triggered hours to days before the onset of the virus specific immune response highlights and it is considered as a one of the first line of defense. It is no surprise, that BVDV has evolved mechanisms that prevent apoptosis and subvert the IFN response. NCP biotypes of BVDV do not induce the synthesis of IFN' s, and cells show no signs of viral infection. Some studies show that NCP BVDV inhibits apoptosis, IFN mRNA and protein synthesis induced by poly(IC), a synthetic form of dsRNA (Schweizer M., *et al*, 2006). By contrast, CP BVDV induce the synthesis of IFN type I and by intrinsic pathway and oxidative stress related mechanisms, activates the kinase cascade, acting as important trigger for apoptosis. At the same time, CP BVDV enhance the production of TNF-α which is an inductor factor of apoptosis (Yamane D., *et al*., 2005).

ANTIGEN PRESENTATION CELLS (APC). APC are pivotal for the induction and control of BVDV immune responses. These cells internalize antigen and present BVDV peptides to T lymphocytes CD4+ (TL helper cells) using the peptide binding site associated with major histocompatibility II molecules (MHC II). In addition, cytokines such as IFN-γ and interleukin 12 (IL-12) and other co-receptor molecules such as B7 are required for adequate presentation and stimulation of T helpers cells.

Even this response, the BVD virus has the ability to cause a reduction of Fc and C3 receptor expression in APC and both, CP and NCP strains, downregulate the MHC II expression in a 30 to 50% (Brackenbury LS., *et al*., 2003; Glew EJ., *et al*., 2003).

EMBRYO RESPONSE TO BVDV INFECTION. The bovine trophoblast has been found to resist infection with BVDV for up to 30-40 days of gestation (Brownlie J., *et al*.,1998; Grooms DL, 2004). This phenomenon is possibly linked to the reported antiviral effect of IFN-τ, a constitutively expressed cytokine of the ruminant trophoblast and its main function is believed to be in the maintenance of the gestation by preventing luteolysis in the ovary of the cow. IFN-τ is not induced by virus and is instead produced constitutively by the embryonic trophectoderm during the period immediately prior to implantation. This type of IFN is produced in very high concentration and has antiviral activity similar to the other type I IFNs, and it may well act to prevent infection of the embryo (Brackenbury LS., *et al*., 2003; Chase CC*., et al,* 2004).

7.2. Adaptative Immune Response

CELLULAR RESPONSE. Lymphocyte T (TL) CD4+ cells play a pivotal role in coordinating a cell-mediated response early in the infection. These CD4+ responses are directed primarily to the BVDV proteins NS3 and E2 and also against the capsid protein (C), glycoprotein E^{rns}, amino-terminal proteinasa (N^{pro}) and nonstructural protein 2-3 (NS2-3) (Pankraz A., *et al*., 2005). The proliferative T response occurs faster with CP than NCP infections, but the CP infections produces approximately twofold higher cell-mediated response (Th1) than NCP. In contrast, NCP BVDV tend to shift the immune response toward the Th2 response and avoid the production of high levels of cell-mediated immunity resulting in a high production levels of antibodies (Chase CC*., et al,* 2004; Lee SR., *et al*., 2009). This fact can explain why the antibody level in NCP BVDV infected dams rises throughout pregnancy and rapidly declines following calving and abortion. The level of antibody in these dams is extremely high by 180 days of pregnancy and it was observed that in dams carrying PI foetuses, the immune response was markedly higher than in those dams carrying uninfected foetuses (Collen T., *et al*., 2000; Smirnova NP., *et al*., 2008).

TL CD8+ cytotoxic play a role in the immune response to acute BVDV infections, producing high levels of IL-2 and IFN-α, indicating a type one memory

response in seropositive cattle. The presence of BVDV has been demonstrated after 9 months post- infection. The expression of MHC class I molecules on infected cells affects the CTL response. In vitro, NCP BVDV causes a 30-40% reduction in MHC class I expression, meanwhile CP BVD virus increase its expression (Collen T., *et al*., 2002).

HUMORAL RESPONSE. The principal antigenic structural polypeptides are the capsid (C), and the glycoprotein glycosylated E^{rns} that although shows to produce significant levels of antibodies, has limited neutralizing activity. Glycoprotein E2 is the major antigen target for antibodies (Chimeno Z.S., *et al*., 2007). E2 is high antigenic and elicits the production of neutralizing antibodies in the host after the vaccination with live or killed vaccines. BVDV type 1 E2 protein has one immunodominant epitope, while BVDV 2 has three. In the case of non structural protein 2-3 (NS23), naturally infected or cattle vaccinated with MLV develop a strong antibody response to this protein, while cattle vaccinated with inactivated vaccines develop little NS23 antibody (Fulton RW., *et al*., 2003).

7.3. Immunosuppression

The changes related to the immunosuppressive action of BVDV include, among others, decreased lymphocyte proliferation and affecting the function of neutrophils, monocytes, macrophages and dendritic cells. In vitro, BVDV-infected monocytes become apoptotic when infected by CP BVDV, and produce soluble factors that induce apoptosis in uninfected monocytes, lympocytes and epithelial cells. This ability may be part of the pathology in lymphoid tissue associated with MD. The field infection with BVDV can results in a 30% to 70% of decrease in the monocyte numbers. BVDV infections have their major effect on thymic and follicular TL. The effect on the number of circulating TL in BVDV infections varies from a mild (10-20%) to severe lymphopenia (50-60%) with highly virulent NCP. BVDV infections causes depletion of both $CD4^+$ and $CD8^+$ TL in the thymus, and there is an overall reduction in TL in the spleen (Piccinini R., *et al,* 2006; Brewoo JN., *et al*., 2007; Shoemaker ML., *et al*., 2008). The different steps of the innate and acquired immunity affected by both CP and NCP BVDV biotypes are presented in the **Tables 1 and 2.**

Table 1. Effects of BVDV on the innate immune system (Chase CC., *et al.*, 2004)

Cellular and / or cytokine changes	Effect on the immune system	Effect on the animal' s disease response
↓ Chemotaxis	↓ Neutrophil migration	↑ Microbial infections
↓ Fc, CD14 and complement receptor expression	↓ Phagocytosis	↑ Microbial infections
↓ Phagocytosis	↓ Ingestion of MO	↑ Microbial infections
↓ TNF-α	↓ Inflammatory cytokine production	↓ Innate and adaptive immune response
↑IL-1 inhibitors	↓Inflammatory and T cell cytokine production	↓ Innate and adaptive immune response
↓ Superoxide production	↑ Microbial growth	↑ Microbial infections
↓ Microbicidal activity	↑ Microbial growth	↑ Microbial infections
↓ IFN production- NCP BVDV	↓ Antiviral response	Immune evasion and viral infection
↑ IFN production- CP BVDV	↑Antiviral response	Apoptosis and death of the infected cells

↑: increased, ↓ : decreased, CD14: LPS receptor, Fc: Immunoglobulin Constant Region Fragment, MO: microorganisms.

8. PATHOLOGICAL FOUNDS

8.1. Macroscopical founds

The pathological founds are confined in several systems. The main clinical and pathological findings associated with the clinical cases of NCP BVDV genotype 2 include widespread mucosal congestion, deep and extensive ulcerations in dorsal and lateral epithelia of the tongue, gums and hard palate. Congestion, multiple haemorrhages and small erosions could be distributed along the mucosa of the oesophagus, pillars of the dorsal sack of the rumen, mucosa of the abomasum and small intestine. Multiple white-yellowish spots diffusely distributed on the omasal mucosa and disseminated areas of congestion and ulcerations covered with fibrin in the small intestine could be observed. Pasty dark red hemorrhagic fluid filling the abomasums, small intestine, and caecum can be found, and the cut surface of the wall of the organs could appeare oedematous. It is common the presence of blood clots of several diameter attached to the mucosa of the ileum, some of them anatomically associated with the Peyer' s patches. In general, the mesenteric lymph nodes are large, oedematous and hemorrhagic (Campbell JR., 2004; Liebler-Tenorio EM., *et al.*, 2006; Lunardi M., *et al.*, 2008).

Interstitial emphysema, pneumonia and fibrinous pleural adherences are commonly found in the respiratory tract. Petechial haemorrhages can be present in epicardium and myocardium. In lymphatic system, several lymph nodes as pre-scapular, scapular, popliteal and mesenteric are hyperplasic. In aborted foetuses, the principal lesions observed include conjunctivitis, pneumonia peribroncheal and non-specific myocarditis. Placental lesions consist mainly in vasculitis, oedema, congestion and haemorrhage with some degeneration and necrosis. Pathological founds in MD are similar than those observed in clinical cases but with severe presentation. Gross examination can reveal digestive tract erosions and ulcerations, often covered with necrotic plaques, including the hard palate, dental pad, tongue, oesophagus and in most of the cases, all digestive tract is involved (Liebler-Tenorio EM., *et al.*, 2003 and 2006).

Table 2. Effects of BVDV on the adaptive immune system (Chase CC., *et al.*, 2004).

Cellular and / or cytokine changes	**Effect on the immune system**	**Effect on the animal' s disease response**
↓ MHC II expression	↓ Antigen presentation	↓ Adaptive immune response
↓ MHC I expression- NCP	↓ CTL-killing	↑ Immune evasion and viral infection
↑ T helper 1 activation- CP	↑ CTL response, T cell memory	↑ Long term protective response
↑T helper 2 activation- NCP	↑ B cell activation	↑ Ab production, ↓ CMI response

↑: increased, ↓ : decreased, Ab: antibody, CMI: Cell Mediated Response, CTL: cytotoxic T-lymphocyte, MHC: Major histocompatibility complex.

8.2. Histophatological founds

The principal microscopic lesions observed in highly virulent NCP BVDV infections reveal severe Lymphocyte T and B depletion and haemorrhages in peripheral and general lymph nodes, meanwhile depletion of lymphoid follicles of Peyer' s patches occurs with both MD and highly virulent NCP BVDV infections. Thymus presents lymphoid depletion in the cortex, and in the tonsils is commune to find follicular hyperplasia and degeneration of germinal centers and necrosis (Odeon AC., *et al.*, 2003; Chase CC. *et al.*, 2004).

In the digestive tract, lymphocyte infiltration, lymphoid depletion and mitosis are the principal lesions. The mesenteric lymph nodes could present hyperplasia of germinal centers, necrosis, lymphoid depletion with mitosis, and eosinophil

granulocyte infiltration. Individual cell necrosis and vacuolation are present in the basal stratum and spinosum stratum of the squamous epithelia of the tongue and oesophagus, and associated with these changes, mononuclear cell reaction (macrophages and lymphocytes) and bacteria colonies could be present. The epithelia of rumen could present cell necrosis and a mild non-suppurative inflammatory reaction. Intestinal lamina propria of the small intestine can have several haemorrhages and moderate inflammatory reaction composed by macrophages, lymphocytes and plasma cells. Also, epithelial degeneration and necrosis are the prominent findings in the small intestine. The affected crypts can be dilated and contained large amounts of cell debris with mixed neutrophils and macrophages (Liebler-Tenorio EM., *et al.*, 2003 and 2006).

In respiratory tract, an acute catarrhal inflammation in nasal cavity and trachea can be observed. The lungs could present moderate congestion and lymphocytic interstitial reaction. Moreover, areas of emphysema with haemorrhages and fibrin clots within emphysematous alveoli, peribronchial lymphoid hyperplasia, focal intralobular interstitial pneumonia, bronchiolitis and focal atelectasis can be observed. The liver could presents focal-subacute inflammations and the kidneys can show a focal interstitial inflammation and proliferation of mesangium cells in the glomeruli. The brain presents perivascular lymphohistiocytic inflammation and panencephalitis. The spleen can show infiltration with neutrophil granulocytes in the red pulp, hyperplasia of Malpighian bodies, necrosis, mitosis and lymphoid depletion (Baule C., *et al.*, 2001).

In aborted foetus, the principal pathological founds are thymus hypoplasia implicated with a morphological immaturity and necrosis and depletion of lymphocytes, attended by infiltration of macrophages. Histopathological changes are also noted in the cerebellum consisting of necrosis and depletion of the external germ layer (Swasdipan S., *et al.*, 2002).

9. DIAGNOSIS

Although BVDV infection can be suspected from clinical signs, the wide range in both diversity and severity makes them the best unreliable for diagnostic investigations. Laboratory tests are mandatory, and should furthermore be used in a planned way to give useful information. In the current BVDV programmes, the aim of the organized diagnostic work is to identify and remove the animals that are the

sources of infection. To achieve this, identification of several categories of animals is necessary (**Table 3**).

9.1. Antigen detection

Virus isolation (VI)

Culture and identification of BVDV from clinical specimens remains the " gold standard" diagnostic technique (Sandvik T., 2005). Since BVDV appears to replicate best in lymphoid cells, samples than contain this cell types should be considered. The samples would include whole blood, buffy coat, lymphoid tissues such as Peyer' s patches, mesenteric lymph nodes, spleen and thymus from post-mortem cattle or aborted foetuses, meanwhile from PI animals, virus can be isolated from blood, serum, buffy coat and a majority of tissues.

BVDV readily grows in many cells lines, however, only three are most widely used in the laboratories for BVDV isolation: bovine turbinate (BT), bovine testicle (Btest) and Madin Darby Bovine Kidney (MDBK) (Sandvik T, 2005; Cornish TE., *et al*., 2005). Foetal calf serum used to supplement the cell culture medium should be free from both BVDV and BVDV-specific antibodies. An incubation period of 4 to 5 days is sufficient for BVDV isolation. However, about 1 in 25 positive BVDV isolations require a second passage. For detection of NCP BVDV in blood from PI animals, cells cultured in 96-well microtitre plates and inoculated with 10-50 µl serum for 4 days may give satisfactory results (Sandvik T., 1995). The growth of BVDV may or may not cause any visible cytophatic effects and most of BVDV isolates (70-90%) are of the NCP biotype. This means that upon completion of virus isolation, cells cultures need to be further tested to detect the presence of NCP BVDV. This is generally performed by fluorescent antibody (FA) staining and Immunoperoxidase (IP) test (Saliki JT., *et al*., 2004; Hilbe M., *et al*., 2007).

For these techniques, the inoculated cells are routinely fixed after 3-5 days. The standard IP assay has proven to be an useful compromise for detection of PI animals in serum, where the virus may be present in concentrations 10^2– 10^3 higher than in acutely infected animals (Muñoz-Zanzi CA., *et al*., 2000). Primary antibodies for the immunostaining can be raised locally, or obtained commercially (panpesti-virus reactive monoclonal antibodies are also suitable). For a given cell line, the sensitivity to infection may decline when it reaches a given passage number. If foetal calf serum is used as medium supplement, it must be free from antibodies to BVDV

as well as the virus itself, otherwise the sensitivity of the IP is compromised. Virus isolation is an essential back-up and reference test for other indirect methods used for identification of PI animals. Unfortunately, VI methods are labour intensive and take several days to be completed, and may not differentiate between TI and PI animals, unless positive cattle are re-tested and remain positive at a later date of 3 weeks (Cornish TE., *et al.*, 2005; Edmondson MA., *et al.,* 2007).

Table 3. Categories of animals in an unvaccinated bovine population where BVDV is prevalent, and usual results of testing for antibodies and virus in serum

CATEGORY	ANTIBODY	VIRUS	COMMENTS
Uninfected, naive animal	-	-	
Acutely infected animal	-	+/-	Brief and low virus titre in blood
Immune animal after acute infection	+	-	
Passively immunized calves	+	-	Antibodies detectable for 5-9 months
PI animals	-	+	
PI calves of immune dams	+	-/+	Antibodies detectable for 4-10 weeks
Mucosal disease cases	-/+	+/-	Neutralizing antibodies
Pregnant cow carrying PI calf	+/++	-	High antibody titre in late pregnancy
Immune bulls	+	-	Semen may be virus positive

Sandvik T, *et al.*, 1999

Antigen Capture Enzymelinked Immunosorbent Assay (ACE-ELISA)

To identify PI animals, VI on primary bovine cells, followed by IP staining is regarded as the "gold standard" method (Saliki JT., *et al.*, 2004). Even the IP test has shown to be highly effective for identifying PI animals, the test is time consuming and requires a high investment both in personal training and laboratory equipment. For that reason, ACEs have been increasingly used since the early 1990's.

The NS2/3-capture ELISA detects BVDV in leukocytes and tissue samples using specific affinity monoclonal antibodies (MAb) against the NS2/3 protein, and has been successfully used to identify PI animals in BVDV control programmes in Norway and in the Shetland islands (Kampa J., *et al.*, 2007). Recently, an ACE ELISA that uses MAbs against the E^{rns} glycoprotein has been developed to detect BVDV. This structural protein is secreted from infected cells during virus replication

and can be detected directly in blood, buffy coat cells, plasma, sera, ear notches or tissue extracts, producing reliable results (Kuhne S., *et al*., 2005; Kennedy JA., *et al*., 2006; Hill FI., *et al.,* 2007). Even this results, the necessity to extract buffy coat cells or process tissue before testing, somewhat limits the applicability to these tests on a large number of samples as requires in whole-herd screening PI animals. In the same way, one of the major complicating factors for viral antigen detection in serum or peripheral blood leukocytes (PBL) is the presence of circulating antibodies (Brodersen BW., 2004), and It is generally accepted that attempts at detection of PI animals less than 3 months of age should not be done due to the presence of colostral antibodies (Cornish TE*., et al.,* 2005).

A commercial kit to detect BVDV antigen in samples as serum and plasma from PI cattle has been used demonstrating good results compared with VI and IHC (Saliki JT., *et al*., 2000; Plavsic MZ., *et al*., 2001). Lately, E^{rns} ACE ELISA has been used as an alternative test due to its high sensitivity and specificity and its use in a precolostral screening (Kampa J., *et al*., 2007). Both samples, serum and skin samples tested with ACE resulted in the correct detection of all cattle identified as PI. Agreement between both ELISAs, performed on serum or skin together with PCR has been reported of 100% (Hill FI., *et al.,* 2007). About ear notches samples, it was reported a sensitivity of 100% and specificity of 99.6% using the commercially available kit on ear tagging obtained from PI animals. The ACE performed on skin provided the greatest ability compared with other diagnostic tests as VI, IHC and RT-PCR to accurately identify animals that were both infected with BVDV (Positive Predictive Value of 100%) and animals truly negative for BVDV (Negative Predictive Value of 100%) (Kennedy JA., *et al*., 2006a and b; Edmonson MA., *et al*., 2007). Ear-notch samples are easy to collect, and preliminary studies have shown that fresh samples are stable, with no reduction in virus detection via ACE and RT-PCR in temperatures of – 20°C, 4°C, and 25°C for 7 days (Ridpath JF., 2003).

In other hand, some researches have reported differences between the results obtained using sera and ear notches samples. Both samples from PI calves were tested using ACE, and while sera samples were negative after intake of colostrum, the ear tissue samples could be detected positive for BVDV all the time. In the same way, testing multiple samples derived from the same ear from PI cattle yielded positive results with low variation (Kuhne S., *et al*., 2005).

Two techniques performed on ear notches, IHC and ACE were compared for detection of BVDV PI animals. Both IHC and ACE detected 100% of PI calves, however, both techniques also detected acutely infected calves, recommending to repeat the test using VI or RT-PCR on buffy coat samples 30 days after initial screening to conclusively discriminate between acute and PI animals (Cornish TE*., et al.,* 2005). Saliki and Dubovi (2004), suggested that acutely infected animals will not have a significant amount of antigen in the skin tissue in the vast majority of cases and that skin samples should be obtained for PI status determination, thus they further suggested that ACE could be primarily useful in screening for PI cattle.

Immunohistochemistry (IHC)

In recent years, BVDV antigen detection in formalin-fixed paraffin-embedded tissues by IHC staining has been widely used for diagnosis of acute infections as well as to detect PI animals (Liebler-Tenorio EM., *et al*., 20004; Loneragan GH., *et al*., 2005; Hilbe M., *et al*., 2007). IHC can be applied on snap-frozen or formalin-fixed paraffin embedded skin biopsies or a wide range of tissues, such as thyroid gland, bone, brain, and mucosal membranes. The distribution of viral antigen in the skin of PI animals has shown to be generalized, but principally is found in aural, neck, brisket, coronary band and tail head skin (Brodersen BW., 2004). Positive IHC staining was shown to be more pronounced in the keratinocytes and in hair follicle epithelium, hair matrix cells of the hair bulb, and the dermal papilla. Generally, IHC staining uses the *15C5* monoclonal antibody, which reacts with the E^{rns} protein of BVDV. These tests exhibit high levels of sensitivity and specificity that are considered adequate for use in screening programs aimed at detecting BVDV infections (Njaa BL., *et al*., 2000).

After the first report by using skin biopsies as method of detection of PI cattle in 1996, where the agreement of IHC and VI in positive and negative animals was of 100% (Thur B., *et al*., 1996), other studies have shown similar results (Njaa BL., *et al*., 2000; Grooms DL., *et al*., 2002). In a study comparing IHC with VI and immunofluorescence (IF) for detection of BVDV in aborted and neonatal calves, the sensitivity and specificity of IHC, IV and IF were 97% and 97%, 83 and 100% and 77% and 88% respectively (Ellis JA., *et al*., 1995). Another study demonstrated IHC testing of formalin-fixed paraffin-embedded tissues had 100% agreement with VI in cases of enteric and respiratory disease and abortion/weak calf syndrome

(Brodersen BW., 2004). Using IHC as the relative gold standard, the serum ACE ELISA had a sensitivity and specificity of 100%, and the real-time RTPCR had a sensitivity of 100% and a specificity of 99% (Hilbe M., *et al*., 2007).

In other hand, when an animal is acutely infected with BVDV or vaccinated with MLV BVDV vaccine, the likelihood of detection of viral antigen in a skin biopsy by IHC is low. This have been demonstrated in several studies where some calves were inoculated with BVDV, being all negative using skin biopsies. Animals experimentally infected with a large inoculum of virulent BVDV can have the antigen in their skin beginning between 8 and 14 days after inoculation, but the duration of which BVDV antigen may be present in the skin of TI cattle is not well characterized (Brodersen BW., 2004). There are some evidences about the different antigen distribution between TI and PI animals, being confined to the epidermal keratinocytes and follicular ostia than in all layers of epidermis, all levels of hair follicles and hair bulb respectively (Shin T., *et al*., 2001). Brodersen BW. *et al*., (2004) also affirmed that skin samples from TI cattle are rarely positive by IHC.

Some of the advantages of this test for detection of PI cattle are the easy sample collection and transport to the laboratory, the reduced costs for ear notch collection, and the stability of the samples comparing with serum or whole blood. Anyway, it is recommended that specimens are transported to the laboratory in a timely manner for improve the results.

Nucleic acid detection

During the past 10 years, the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) has gained widespread use as a routine diagnostic method for BVDV (Smith RL., *et al*., 2008). The RT-PCR test, a relatively new diagnostic tool for demonstrating the presence of BVDV antigen has been used to detect infected and PI animals with BVDV, and theoretically, every imaginable animal specimen like milk, urine, tissue, serum, whole blood, buffy coat cells, swabs, skin, formalin-fixed tissues, etc., can be used (Drew TW., *et al*., 1999; Young NJ., *et al*., 2006). Lately, the use of oral swabs samples has been described as a useful method for screening and detection of BVDV PI cattle by RT-PCR (Tajima M., *et al*., 2008).

Detection of *Pestivirus* RNA by RT-PCR includes four different steps: extraction of RNA, reverse transcription to cDNA, primer-directed amplification and lastly detection of amplified products. Lately, with the new Real-Time PCR in a

single tube, the need of gel electrophoresis and the risk for carry-over contamination with previously amplified DNA are eliminated (Young NJ., *et al*., 2006). When complete genomic sequences of viruses from the *Pestivirus* genus are compared, the highest level of conservation is observed in the 5' UTR, and for that reason, RT-PCR assays using primers specific for this region have offered the best epidemiological sensitivity. At the same time, sequences variations in this region are conserved among viruses within the same *Pestiviral* genotype, permitting their differentiation (Letellier C., *et al*., 1999; Vilcek S., *et al*., 1998).

Cornish TE., *et al*. (2005) reported a complete agreement among IHC and ACE from skin samples, VI from white blood cells (WBC) lysates, and RT-PCR from WBC lysates from PI calves with BVDV. In the same way, using IHC as the relative gold standard, the RT-PCR has shown a sensitivity and specificity of 100% and 99% respectively (Hilbe M., *et al*., 2007). Additionally, RT-PCR test is a reliable way of diagnosing PI calves at all ages and it has been demonstrated that RT-PCR is able to detect BVDV even in the presence of colostral antibodies (Letellier C., *et al*., 1999; Luzzago C., *et al*. 2001; Saliki JT., *et al*., 2004; Sandvik T., 2005). Another useful diagnostic application of the RT-PCR method is monitoring of cell cultures and foetal calf serum used as cell culture medium supplement, both of which may be contaminated with NCP BVDV (Bolin SR., *et al*., 1991).

More recently, Real-Time PCR is being used to identify, classify and quantify many viral pathogens as it is a highly sensitive and rapid method for detecting viral nucleic acid sequences in clinical specimens. Real-time RT-PCR methods for genotyping BVDV have been described previously, using separate sets of primers and probes in the same assay (Lettellier and Kerkhofs, 2003). Real-time PCR offers the same advantages as conventional PCR assays, but is considered as a faster method due to real-time visualization of results (Baxi M., *et al*., 2006). When Real Time RT-PCR assay was compared with virus isolation in sera samples from PI animals, there was full agreement between both tests. Thus, the one-step Real-Time RT-PCR assay appears to be a rapid, sensitive, and specific test for detection and typing of BVDV (Baxi M., *et al*., 2006). In an other study, The TaqMan® RT-PCR assay was 10 times more sensitive than a gel-based RT-PCR, distinguishing three positive samples in heparin-treated whole blood that conventional RT-PCR failed to detect (Drew TW., *et al*., 1999).

RT-PCR and Real-Time PCR have become a sensitive and specific diagnostic methods for testing pooled samples as serum, milk, blood and supernatant from skin biopsy samples, and they have been evaluated as a low-cost alternative for diagnosis screening and surveillance of persistent infections (Kennedy JA., *et al*., 2006 a and b; Muň oz-Zanzi C.,*et a*l., 2006). When determining the size of the pool, the pooling protocol should use the fewest number of tests required to identify all animals PI with BVDV in a herd. A simulation model for determining the economically optimum sample size in populations with various prevalences of PI found that the economically optimum sample size is dependent upon the prevalence of true positives in the population. For a PI prevalence of 0.5% to 1.0%, the optimum number of samples in an initial pool is 20 to 30, and as prevalence increases the least-cost initial pool size decreases. If the pool size is too large, there is an increased risk that any single pool will test positive, requiring additional testing to identify the small number of viremic animals in the positive pool. If the samples are grouped in unnecessarily small pools, the cost benefit of pooling samples is lost to the large number of negative pools tested for each positive pool identified (Weinstock D., *et al*., 2001; Larson LR., *et al.*, 2005, Muň oz-Zanzi C.,*et a*l., 2006).

For an average prevalence of PI animals with BVDV of 0.01 and viremia in each animal between 10^2 and 10^7 virus particles/ml, the pool size associated with the lowest number of tests and cost corresponds to eight samples/pool (Muň oz-Zanzi C.,*et a*l., 2006). The use of RT-PCR on pooled ear-notch supernatant could provide an initial, rapid, cost-effective method of screening cattle herds for BVDV PI animals. Subsequent serial testing with an ACE to evaluate individual samples included in the positive pool could minimize the length of time other animals are exposed to the virus. Using this procedure, a sensitivity and specificity of 100% and 97.5% respectively has been reported (Kennedy JA., *et al*., 2006 a and b).

9.2 Antibody detection

Studies aimed on immune response against BVDV have been concentrated on the demonstration of the presence of antibodies against the virus, which can be detected in serum from 3 weeks, and thereafter for years after acute infection in immunocompetent animals. Antibodies to viral glycoproteins (primarily E2) may neutralize the virus and can cross react with other strains of BVDV, but if sera raised against one virus strain are tested against other challenge viruses, different

antibodies titres may be obtained. Conversely, the highly immunogenic non structural viral protein NS2-3, which is essential for the intracellular replication of the virus, is antigenically conserved between all pestiviruses (Sandvik T., 1995; Kuijk H., *et al.*, 2008).

Virus Neutralization test (VN)

This assay is sensitive and specific for detection of antibodies to BVDV, and it has been recognized as the reference test for BVDV serology (Seki Y., *et al.*, 2007). The VN test is a biological in vitro system, which quantifies the inhibitory effect of specific antibodies on virus replication in cell cultures. The test is usually performed in 96-well microtitre plates, where serially diluted sera are incubated with a CP challenge virus before susceptible bovine cells are added, for finally incubated with the neutralized virus for 4 days (Schefers J., *et al.*, 2008). The antibodies detected by VN are predominantly against E2, which may result in different titres depending on which virus strain is used in the assay. Because of the high specificity of the test, it is essential to use a challenge virus antigenically similar to the field viruses in the population to be tested. Established reference strains such as the NADL or Oregon C24V are both BVDV-1a, and if the predominating field virus strains differ antigenically from these, a cytopathogenic local virus isolate may be a better choice if a high sensitivity is desired. In addition, a BVDV-2 virus may be useful as a second VN challenge virus, since it represents the antigenically most distantly related BVDV. When calibrated properly, VN is a very specific and sensitive assay. However, it is labour intensive, requires substantial investments in selection and monitoring of cells cultures and media to give satisfactory test results, and therefore, it is not suitable for examination of few or sporadic samples (Sandvik T., 2005; Seki Y., *et al.*, 2007).

Enzymed-Linked Immunosorbent Assay (ELISA)

The measurement of antibody responses of animals exposed to BVDV either through a natural exposure or an immunization protocol is still a standard procedure. Several ELISAs for detection of BVDV specific antibodies have been available commercially since the early 1990s. For BVDV serology they have become popular for several reasons: they are independent from cell cultures, they can easily be applied for mass screening and results can be read in few hours (Sandvik T., 2005). Generally, It has been shown that the prevalence of seropositive animals in herds

with one or more PI animals is high, ranging from 53 % to 98% (Waldner CL., *et al.,* 2005) compared with those herds without PI animals (Thobokwe G., *et al*., 2004).

For studies at the animal level, most ELISAs have been developed for the detection of antibodies to BVDV in serum. However, for screening non-vaccinated herds, ELISAs for antibody detection in bulk tank milk have been the method of choice, being the most economic procedure available for milk herd screening and reporting a sensitivity and specificity of 97.9 and 99.7%, respectively (Greiser-Wilke I., *et al*, 2003; Solis-Calderon JJ., *et al*., 2005). An ELISA based on a monoclonal antibody against non-structural protein NS2-3 using milk samples has shown a sensitivity and specificity of 95 and 97.7% respectively (Beaudeau F., *et al*., 2001).

ELISA to detect BVDV in bulk milk has been recognized as a valuable tool to estimate the prevalence of infected animals in the concerned herd, and consequently, to identify both BVDV-free dairy herds and herds suspected of harbouring an active infection (Niskanen R., 1993). This methodology is currently used to identify uninfected herds in the BVDV control and eradication programmes developed in Scandinavian countries (Lindberg and Alenius, 1999). High absorbance values obtained have been correlated with the prevalence of BVDV-positive cows in a herd, obtaining four groups of herds: Herd classes 0 and 1 (characterized by low OD values) which represent herds with absence of infection and herds without recent contact with PI animals, and Herd classes 2 and 3 (high OD values) that represent herds with recent contact with PI animals and active infection and herds with the presence of lactating PI animals respectively. For the identification of the single PI animals in Herd classes 2 and 3, individual serum samples have to be analyzed by ACE or RT-PCR (Greiser-Wilke I., *et al*., 2003).

In the **Table 4** are present the different tests that could be used in different situations of infection in a given herd.

9.3. Role of the maternal antibodies in the detection of PI animals

When maternal antibodies to BVD virus are present in the blood of PI calves, VI carried out on peripheral blood or on post-mortem specimens may be falsely negative (Palfi V., *et al*., 1993). A predictive study to help to define the proper age to avoid BVDV maternal antibody interference, estimates that it took calves 141 days to become seronegative from BVDV type 1 antibodies and 114 days for BVDV type 2. Colostrum may contain virus neutralizing antibodies with titres higher than

1:500,000 and those antibodies may interfere with BVDV antigen detection (Endsley JJ., *et al*., 2003). Some results clearly show that a high proportion of PI animals tested during the first week after the ingestion of colostrum with VI and ACE are negative. There is a clear relationship with the maternal antibody status, thus, in the presence of high levels of maternal antibodies, the VI and the ACE tests are unreliable indicators of the presence of persistent infections with BVD virus (Palfi V., *et al*., 1993). However, the RT-PCR test gave positive results even in the presence of high antibody titres, demonstrating the suitability of this test for use in eradication programs (Zimmer GM., *et al*., 2004).

9.4. Screening acute infections

For animals that are still alive, the most recommendable sample to detect BVDV is whole blood (Saliki JT., et *al*., 2004). In acute infections, detectable virus remain in mononuclear cells two or three times longer than in serum. To be successful using serum from an acutely infected animal, the sample would need to be collected 3 to 8 days post-infection. Because the date of infection is rarely known, this narrow range of viremia makes difficult to establish the best time for sampling. Swabs of mucosal surfaces can also be a successful sample for detecting BVDV, particularly in early phase of infection (Tajima M., *et al*., 2008). Paired serum samples for measuring the titer of neutralizing antibodies is still the current norm. Sampling of non affected animals for comparison is often recommended, particularly when no previous testing in the herd has been carried out.

If clinical signs are due to the MD syndrome, the sampling recommendations for acutely infected animal are still valid. Because a true case of MD begins with a PI animal, virus is present in virtually any sample collected (Luzzago C., et al., 2006; Hilbe M., *et al*., 2007a). Ear notches should be collected from affected animals to define the disease status. In the vast majority of cases, acutely infected animals will not have a significant amount of antigen in the skin tissue (Walz PH., *et al*., 2001; Brodersen BW., 2004).

9.5. Screening persistent infections

The PI animals represent less than 1% of the bovine population (Brock KV, 2003; Campbell JR., 2004). Some possible testing scenarios are present in **Table 5**.

Table 4. Suggested Diagnostic Laboratory tests for given testing situations (Larson RL., *et al.*, 2005)

SITUATION	TEST	RATIONALE
Testing sick suckling calves (pneumonia, septicaemia, etc.) for possible BVDV involvement	*IHC or ACE for skin samples to identify PI and sometimes TI animals *PCR of blood or serum to identify PI and TI animals	*Maternal Antibodies may interfere with VI and ACE using serum or plasma. *If negativity is observed in ACE or IHC and positivity in PCR from blood or serum, TI infection is likely.
Testing dead suckling calves for BVDV possible involvement	*IHC or ACE for skin samples to identify PI and sometimes TI animals *IHC, FA or VI from tissues to identify infected calves (no difference between PI and TI animals)	*Maternal Antibodies may interfere with VI and ACE-ELISA using serum or plasma. *If a dead calf is IHC or ACE negative from skin sample, but positive from a tissue sample, TI is likely.
Screening a herd (suckling calves, cows that lost calves, replacement animals) because there is laboratory evidence of BVDV in the herd	*IHC or ACE for skin samples to identify PI and sometimes TI animals	*Maternal Antibodies may interfere with VI and ACE-ELISA using serum or plasma.
Screening open replacement heifers (raised or purchased), purchased open cows, or bulls raised or purchased	*IHC or ACE for skin samples to identify PI and sometimes TI animals *PCR- pool serum or whole blood into groups of 30-40 or less. Test individual skin samples of animals in positive pools to identify PI' s. Animals in negative pools are considered not-PI.	Any positive result could be confirmed by using IHC, ACE, VI or PCR of serum or blood samples taken no less than 21 days later.
Screening purchased pregnant replacement heifers or cows prior to entry into the herd	*IHC or ACE for skin samples to identify PI and sometimes TI animals *PCR- pool serum or whole blood into groups of 30-40 or less. Test individual skin samples of animals in positive pools to identify PI' s. Animals in negative pools are considered not-PI. *Isolate pregnant animals away from resident herd until the calf is born and tested for PI status via IHC or ACE from skin samples.	Any positive result could be confirmed by using IHC, ACE, VI or PCR of serum or blood samples taken no less than 21 days later.
Screening raised replacements heifers and bulls prior to sale by a supplier	*IHC or ACE for skin samples to identify PI and sometimes TI animals *PCR- pool serum or whole blood into groups of 30-40 or less. Test individual skin samples of animals in positive pools to identify PI' s. Animals in negative pools are considered not-PI.	Any positive result could be confirmed by using IHC, ACE, VI or PCR of serum or blood samples taken no less than 21 days later.

Table 5. Suggested diagnostic laboratory tests for the detection of PI animals (Larson RL., *et al*, 2004)

TEST	COST	ADVANTAGES	DISVANTAGES	SPECIMENS/SHIPPING
Virus isolation 1-3 week turnaround	Moderate to high cost	-Gold standard for BVDV -High specificity -Virus is available for study at a later date	-Slow procedure -Labor-intensive -Potential false negative due to interference by maternal Ab -Retest positive animals in 3-4 weeks to distinguish between PI and TI	-Whole blood (10 m) or serum (2-3 ml) -Send in container with cold packs. -Do not freeze the samples
Immunohistochemistry (IHC) 2-5 day turnaround	Low cost	-High sensitivity -Usually identifies only PI TI usually test negative	-Labour-intensive -Formaline usage -Will not generally identify TI animals	-Skin samples-ear notch -Send fresh on wet ice or stored in 1:10 volume of 10% neutral buffered formalin -Sample can be held in formalin for several weeks
Antigen-Caputure ELISA of serum 1-2 day turnaround	Low cost	-High sensitivity -Easy to carry out	-Potential false negative due to the interference by maternal antibodies -Variation of viremia -To distinguish between PI and TI animals, retest 3 weeks later	-Serum (2 ml) -Send in insulated container with cold packs.
Antigen-Caputure ELISA of skin 1-2 day turnaround	Low cost	-High sensitivity -Usually identifies only PI TI animals usually test negative	-Will generally not identify TI animals	-Skin samples-ear notches -Send in insulated container with cold packs. -Do not allow to dry out
Antigen-Caputure ELISA of tissue / leukocytes 1-3 day turnaround	Low cost	-High sensitivity	-Labor-intensive to prepare buffy coat -Not used in a large screening	-Whole blood (10 ml) using EDTA or heparin -Tissues -Send in insulted container with cold packs.
Polymerase chain reaction (PCR) 1-3 day turnaround	-Moderate to high cost (can be reduced pooling samples)	-High sensitivity Can detect 1 ng/ml BVDV RNA	-Potential of false positive due to laboratory contamination -Retest samples in 3 weeks to distinguish between PI and TI animals	-Whole blood (10 ml) or serum (2-3 ml) -ear noches in red top tubes -milk, semen and tissues -Send in insulted container with cold packs.

10. CONTROL

A general model for successful BVDV control is proposed in **Fig. 8**. Three necessary elements are identified: bio-security, virus elimination and monitoring (Lindberg A. and Houe H., 2005).

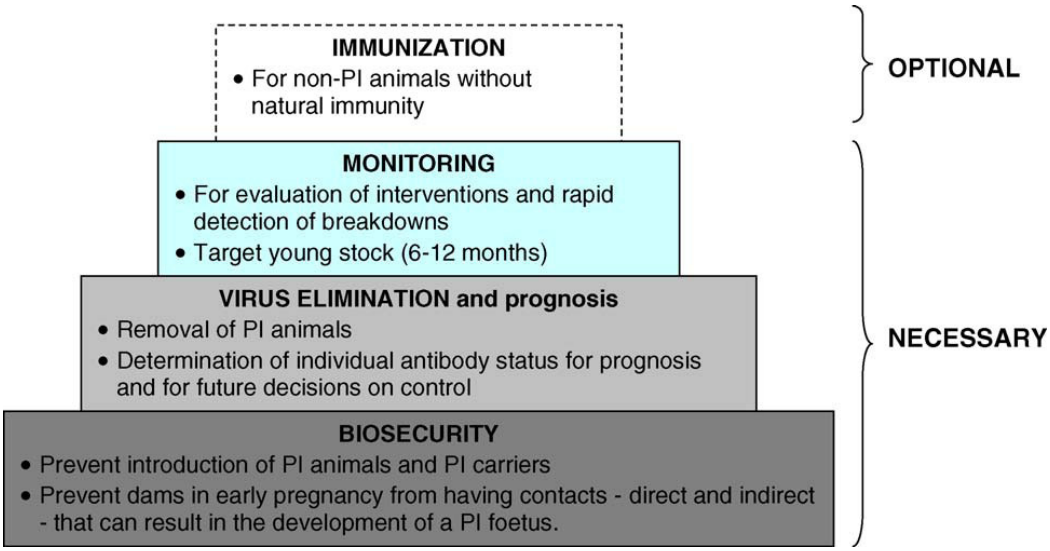


Fig. 8. General model for systematic control of BVDV. Bio-security, virus elimination and monitoring are necessary

In the context of BVDV control it is helpful to distinguish between non-systematic and systematic approaches. After the pivotal role of PI animals for the perpetuation of BVDV infection had been recognised, the removal of these animals from infected herd became the most important control (Ellis JA., *et al.*, 1995; Hilbe M., *et al.*, 2007). However, as long as vaccination and removal strategies are being used exclusively on herd basis without systematic follow-up or monitoring the outcome, they can only be described as non-systematic. In contrast, systematic control implies a goal-oriented reduction in the incidence and prevalence of BVDV infections, typically implemented on a regional or national level (Reichel MP., *et al.,* 2008).

10.1. Non systematic control -Vaccination

For a long time, the purpose of vaccination was the prevention of acute clinical diseases in herds. The crucial issue of breaking the infectious cycle by preventing the generation of new PI animals was long neglected or not even addressed. Only in the 1990ies the importance of intrauterine protection as a primary goal of vaccination

became clear. In many countries vaccination is widely used and many different vaccines are available. However, there is no indication that vaccination alone has ever resulted in decline of BVDV prevalence. In summary, non-systematic control efforts may well result in improved animal health and prevent BVDV associated losses. However, their efficacy is mostly restricted to the herd level, usually for a limited time period, and no decrease of the overall BVDV prevalence can be expected (Greiser-Wilke I., *et al.*, 2003; Moennig V., *et al.*, 2006; Houe H., *et al.*, 2006).

10.2. Systematic control

The first systematic control/eradication schemes were successfully developed in Scandinavian countries in the early 1990s. Later, some other European countries or regions followed by Scandinavian example by implementing similar control regimes for the control of BVDV. The identification and removal of PI animals as well as the prevention of new infectious with BVDV are key elements of this kind of control and could be developed with and without vaccination (Ståhl K., *et al.*, 2005; Hult L., *et al.*, 2005; Houe H., *et al.*, 2006).

Systematic control without vaccination

Initial test for classification of herd status:

- Implementation of additional biosecurity measures.
- Follow-up tests for identification and removal of individual infected animals in infected herds.
- Continued monitoring of supposedly free herds to confirm a free status and/or for rapid detection of any new infection.

In a non-vaccination environment a herd diagnosis can be performed by assessing the serological situation. The goal is to detect the presence or absence of antibodies against BVDV. The diagnosis can be based on testing antibodies in bulk milk, in individual or pooled serum samples from young stock or in pooled samples (milk or serum) from primiparous cows and the virus detection in bulk milk (by PCR) (Mars MH., *et al.*, 2005).

The first systematic programmes aimed at eradicating BVDV without the use of vaccines were launched in 1993-1994 in Denmark, Finland, Norway and Sweden.

Despite different preconditions in terms of initial prevalence of herd with PI animals varying from 1% in Finland to 50% in Denmark it has taken all countries approximately 10 years to reach their final phases. First to reach eradication will most likely be Norway (Rikula U., *et al*., 2005). Austria has followed the Scandinavian example and after seven years, approximately 30% of them targeted herds are certified as being free for BVDV (Rossmanith W., *et al*., 2005).

Systematic control efforts have also been implemented in other parts of Europe, such as on the Shetland Islands were BVDV has been eradicated, and in Brittany (France), in The Netherlands (Mars MH., *et al*., 2005) and Germany (Moennig V., *et al*., 2005). Time-limited, project type control efforts have been implemented in the Rome area, and in the Lecco and Como regions of Italy (Ferrari G., *et al*., 1999; Luzzago C., *et al*., 2001). It should be noted that the only approaches that emphasis on biosecurity in general, and control of direct animals contacts in particular with or without the complementary use of vaccines.

In a given herd under systematic protocol, a BVDV biosecurity guidelines on how to avoid re-infection relevant to the cattle population in question is necessary. It is important to identify and remove PI animals as fast as possible in order to reduce the risk of transmission (if it is possible, testing them before colostrum is given). Undetected PI animals are thus a source of new infections and it is essential to avoid the contact between susceptible dams in early pregnancy and PI animals. A common source or re-infection is accidental reintroduction from infected herds. If pregnant replacement animals of unknown BVDV status are to be introduced into a herd cleared of BVDV, they should be kept in quarantine facilities until they are verified free from the disease. Other means of infection are biologic products, including semen, embryos, colostrum, vaccines and other veterinarian drugs used. Similar visitors clothing, equipment or other fomites should be monitored with regard to possible BVDV contamination. Recommendations on use of disinfectants should be readily available to farmers (Houe H., 1995; Sandvik T., 2004; Smith DR., *et al*., 2004; Luzzago C., *et al.,* 2008).

Systematic control with vaccination

Under circumstances where the Scandinavian approach of eradication might not be applicable for several reasons, systematic vaccination after removal of PI animals

is an option in order to minimize the risk of re-introduction of the virus into cleared herds. The programme comprises the following elements (Moennig V., *et al.*, 2006):

- Initial test for classification of herd status, identification and removal of PI animals.
- Follow-up tests for identification and removal of PI calves born within the first 12 months after clearance of infected herds.
- Systematic vaccination using a vaccine and protocol with a proven record to protect pregnant animals against foetal infection.
- General and individual biosecurity measures.

Since vaccination might interfere with bulk milk testing, initial screening of herds depends on the epidemiological situation. In vaccinated herds, a few animals older than 6 months should be kept unvaccinated so they can work as sentinel animals for indications the infection pressure in the herd (Moennig V., *et al.*, 2005a). The option to identify PI animals in dairy herds could be the PCR in milk samples and in positive cases, PI animals will be identified individually. The rest of the herd can be tested either using individual or pooled blood samples or ear notch samples that will be analysed using ACE or RT-PCR (Mars MH., *et al.*, 2005; Kennedy JA., *et al.*, 2006 a and b).

When all PI animals have been removed, systematic vaccination, preferably a two step control is implemented. Depending on the epidemiological situation, vaccination can be mandatory or banned. In areas with high density of cattle, intense trade, sub-optimal control of biosecurity and a high prevalence of BVDV, systematic vaccination should be used until the incidence of PI cattle has reached a low level. In areas with low BVDV prevalence, vaccination should be banned because the risk of re-introduction of BVDV into free herds is likely to be low. In any case, continued monitoring is an essential element of the program (Lindberg A., *et al.*, 2006; Kalaycioglu AT., 2007).

Finally, the predictors for the efficiency with which control measures can systematically reduce the prevalence of BVDV infections are: the ability to prevent new infections, the ability to rapidly detect new cases of infection, the ability to intervene in infected herds (efficiency of diagnostic), strength in incentive to clear infected herds, the ability to get acceptance of/compliance of the programme,

collaboration between the farmer' s organizations, strength in support from authorities through legislation and impact of market effects (Lindberg A., *et al.*, 2005; Moennig V., *et al.*, 2006).

11. PROFILAXIS

BVDV vaccines are important component of BVDV prevention and control programs. For decades, the majority of vaccines contained only biotype 1 BVDV, but due to the widening diversity of field BVDV strains, the animal health industry has responded in two ways: adding both, 1 and 2 BVDV genotypes to vaccines, or performing cross-protection studies with vaccines containing a type 1 vaccines (Kelling CL., 2004). Because BVDV vaccines should protect against systemic infection with a range of antigenic variant strains of BVDV isolates, some important variables to consider when selecting a vaccine include the immune response, crossreactivity, fetal protection, duration of immunity, immunosuppression, reversion to virulence and effect of maternal antibody on immune response and purity (Chase CC., *et al.*, 20004; Platt R., *et al.*, 2008).

11.1. Modified-lived virus vaccines (MLV)

The major advantage of MLV is the stimulation and production of relatively high concentrations of viral neutralizing antibodies, correlating with protection in future exposure with virulent virus (Ellsworth MA., *et al.*, 2003). Generally, MLV actives all phases of the immune system, yielding a balanced systemic and local immune response and a balanced humoral and cell-mediated response (Reber AJ., *et al.*, 2006). Some studies have shown that vaccinating with a MLV using both BVDV genotypes, it is possible to increase cell-mediated immune memory response, meanwhile others researches report that the vaccination only with BVDV type 1 provide cross-protection against type 2 BVDV challenges. Crossreactivity of antibody induced in response to MLV BVDV vaccines was demonstrated, where vaccinated cattle produced antibodies by three weeks that neutralized different strains of BVDV in vitro (Fulton RW., *et al.*, 2003). In the same way, experimental challenge-exposure experiments have been conducted to confirm that MLV type 1 vaccines crossprotect young calves from experimental infection with a virulent strain of BVDV type 2 (Kelling CL., *et al.*, 2004 and 2007). In contrast, some studies have demonstrated that CP BVDV type 1a failed to provide type 2 BVDV protection. In

some way, some cross protection is afforded by BVDV single strain, but the wide antigenic diversity and different clinical syndromes of BVDV strains requires the use of more than a single strain to develop high levels of protection on the field (Dean HJ., *et al*., 1999).

Stressed cattle should not be vaccinated with MLV BVDV vaccines, because this vaccines in some cases can induce prolonged suppression on the host defense mechanisms. Features as the potential risk of shedding BVDV after vaccination with MLV, followed by the transmission to susceptible contact animals and the reversion to virulence have been examined experimentally. In one study, calves vaccinated with MVL develop a transient viremia and nasal secretion for several days (Kelling CL., 2004). The maternal antibodies in calves play an important role on their efficacy when they are present in high concentrations, due to the block of an adequate protective immune response (Zimmer GM., *et al*., 2004).

11.2. Inactivated vaccines

The major advantage of inactivated BVDV vaccines is that they are safe, do not produce immunosuppressive effects and they are not fetopathogenics. The principal disvantages are that they generally induce a weaker neutralizing antibody response, a shorter duration of protection and a limited cytotoxic T-cell response, which plays an important role in recovery and resistance to disease (Makoschey B., *et al*., 2001; Platt R., *et al*., 2008). In the case of inactivated BVDV vaccines, they have shown to confer high protection only to the specific strain used and a very low response to different genotypes. Some studies have shown that inactivated type 1a vaccines provided only 78% of protection against a type 1a challenge, 63% of protection against 1b type challenge and no protection against BVDV type 2 challenge.

11.3. DNA and recombinant vaccines

Lately, DNA and recombinant viral vector vaccines have been created and tested on an experimental level to develop a vaccination strategy to overcome the shortcomings of MLV and inactivated vaccines. BVDV vaccines encoding the type 1a E2 protein have been tested in mice and cattle, and they induce the formation of neutralizing antibodies and cell-mediated immune response, followed by B-cell memory, but unfortunately they have offered limited protection in cattle challenged

with an heterologous BVDV type 1b strain. Recombinant BVDV immunogens expressing structural glycoproteins E0, E1 and E2 have been produced and tested in cattle, observing a high induction of neutralizing antibodies against the homologous strain as well as against heterologous strains in a very efficient way (Chimeno ZS., *et al*., 2007). In other hand, calves immunized with a plasmid encoding either BVDV type 1 and 2 E2 glycoprotein and consecutively challenged with BVDV-2 strain, developed both humoral and cell-mediated immune responses, including virus-neutralizing antibodies and IFN-gamma-secreting cells in the peripheral blood. Additionally, animals presented little leucopenia and no weight loss or temperature response (Young NJ., *et al.,* 2007; Liang R., *et al*., 2008).

11.4. Foetal protection

The principal goal to improve the vaccination scheme in the cattle is the prevention of the developing fetuses infected with BVDV, principally PI animals (Ellsworth MA., *et al*., 2006). Foetal protection with two different MVL vaccines containing single type 1 BVDV provided protection that varied from 83 to 92% against type1 BVDV challenge, but only 58% against type 2 challenge (Kovács F., *et al*., 2003; Brock KV., *et al*., 2006). This lack of strong cross protection has been also seen in field cases. In other hand, using a MLV vaccine containing two BVDV strains type 1a (Singer) and type 2, it was possible to observe a foetal protection of 100% in the challenge with type 1, and 95% against type 2 challenge (Chase CC., *et al*., 2004). In a study aimed on the determination of the efficacy of an inactivated BVDV vaccine in preventing fetal infection, it was obswerved that 36% of fetuses from dams vaccinated pre-breeding with inactivated vaccine, and experimentally challenge-exposed between 80 and 90 days of gestation, were protected against BVDV infection. Inactivated vaccines should contain more than a single strain to develop cross reaction in the field (Zimmer GM., *et al*., 2002; Kellying CL, 2004).

In contrast, using inactivated BVDV vaccine followed by vaccination with MVL BVDV vaccine, it was established that heifers intranasally challenged 4 weeks after the second vaccination (between 30 and 120 days of gestation) with a mixture of type 1 and type 2 BVDV isolates, delivered clinically healthy seronegative and BVDV-free calves (Greiser-Wilke I., *et al*., 2003). After these researches, a " Two-steps" vaccination is recommended and available everywhere (Moennig V., *et al*., 2005). First vaccination is carry out using and inactivated vaccine, and four weeks

later a booster vaccination is applied using a MLV. This protocol ensures a long-lasting immune response and a comprehensive foetal protection. It has never been observed that MLV virus is shed when used in the context of two-step vaccination. Animals must be vaccinated at the latest eight weeks before their first pregnancy. Authors suggest that a group of young animals older than 6 months could kept unvaccinated and monitored for BVDV antibodies to confirm that there is no new introduction of infection (Frey HR., *et al*., 2002; Oguzoglu TC., *et al*., 2003).

A general vaccination program can be implemented in healthy replacement heifers (isolated from pregnant cows) based on the twice administration of BVDV MLV before breeding. Vaccination should be timed so that maximal protection is afforded during the critical first 4 months of gestation to maximize the potential of adequate duration of immunity. MLV should be administrated 60 days before breeding. If inactivated vaccines are used instead of MLV products, vaccination of heifers before breeding should be timed so that maximal response are achieved. The second dose of the primary series should be administrated 2 weeks before breeding. Cows should be revaccinate annually, principally 2 weeks before breeding (Kellying CL., 2004).

II. FIRST EXPERIMENTAL PHASE

1. INTRODUCTION

BVDV is a significant pathogen associated with gastrointestinal, respiratory, and reproductive diseases of cattle worldwide. The ability of BVDV to cross the placenta during early pregnancy can result in the birth of PI calves, which are capable of shedding large quantities of virus throughout their lives and are considered the primary reservoirs for BVDV. For this reason, the detection of the principal sources of infection (PI animals) is the important key in the control of the disease spreading (Kelling CL., 2004; Lindberg A. and Houe H., 2005). PI animals do not show antibody response and diagnostic tests such as virus isolation, IHC, Antigen-Capture ELISA, and RT-PCR are used in their detection (Cornish TE., *et al.*, 2005; Sandvik T., 2005). Control efforts based on the identification and elimination of PI animals have been successful in a number of countries around the world. In Italy, little research has been done about BVDV seroprevalence and at this time no compulsory/voluntary control program exists in order to detect and eliminate PI animals. BVDV-1 is the predominant and most wide distributed genotype in the north of the country, where 30% of the bovine population is concentred. In particular, BVDV-1b is the most frequently isolated, followed by groups c, d and e (Luzzago C. *et al.*, 2001; Falcone E. *et al.*, 2001 and 2003). In contrast, BVDV-2 genotype has been isolated from 2 healthy animals from a farm in the northern Italy where a several outbreak originated for the use of Herpesvirus-1 MLV contaminated with BVDV-2 strain (Falcone E. *et al.*, 1999; Luzzago C. *et al.*, 2001). The importance to study the seroprevalence in central regions and the identification and segregation of BVDV genotypes that are present in infected and PI animals in Italy, could be a significant research to understand the distribution and the movement of the virus in the different regions of the country, and also can contribute with important information to understand the epidemiology of the disease aimed to future control programs that could be developed in this country.

2. Study design

The aim of this study was to compare the different techniques used routinely in the detection of BVDV infected and PI animals. Following that objective, the first step was to contact cattle farms that were agree to collaborate in a voluntary BVDV eradication program based on the detection and elimination of PI animals. In the farms included in the study, an important anamnesis was carried out. Sera samples

were obtained from animals in each herd and were tested using a commercial *Antibody BVDV ELISA* kit in order to detect the presence of antibodies against BVDV. Seropositive animals were considered infected (if not vaccinated) or with a post-vaccination immune response, being excluded as PI candidates. In other hand, all seronegative and young animals (< 2 years old) were considered as possible PI, and were tested to detect BVDV antigen in sera samples with a commercial kit *Serum/Antigen-Capture ELISA* (*serum/ACE*). The criteria of choosing animals younger than 2 years old is because PI animals are susceptible to suffer secondary diseases, and generally die before reach 2 years old. In order to standardize a RT-PCR protocol, RNA from four BVDV infected cell-cultures (one genotype 1 and three genotype 2) were used as positive controls. Three PCRs were standardized using specific primers that amplifies a high conserved region of the 5' UTR, one targeting the Pes*tivirus* genus and two for differentiate between BVDV genotypes 1 and 2. Sera samples from seronegative and young animals were pooled (5 samples/pool) and tested with the RT-PCR standardized, and if a positive pool was obtained, samples of the pool were tested individually. Finally, If seronegative/ antigen positive animals were detected, they were inserted in a second sampling plan consisting in buffy coat, ear notch and tissues samples (if animals were culled) for being tested with other techniques as *Leukocyte/ACE*, IHC and RT-PCR.

3. Farms and animals sampled

An epidemiological form was created (***Annex 1***) based on different researches that have measured the principal risk factors associated with the presentation of the BVDV and the presence of PI animals in farms in different countries (Luzzago C. *et al.*, 1999; Ferrari G. *et al.*, 1999; Nuotio L. *et al.*, 1999; Biuk-Rudan N. *et al.*, 1999; Obando C. *et al.*, 1999; Mainar-Jaime RC. *et al.*, 2001; VanLeeuwen JA. *et al.*, 2001; Stahl K., *et al.*, 2002; Mockeliuniene V. *et al.*, 2004; Solis-Calderón JJ. *et al.*, 2005; Niza-Ribeiro J. *et al.*, 2005). For a better understanding of the dynamic of BVDV infection and for the possibility to identify recent infections within farms, animals were categorized by age (in months) in two groups: younger and older than 24 months old. In general, if young animals are infected, there is a high possibility that some PI animals are spreading the disease and causing recent or on-going infections in the farm. In the same way, another important and useful information as

sex, breed, clinical history of the farm and introduction or movement of animals inside the farms was collected.

A total number of 21 farms were sampled. Twenty were unvaccinated farms (10 were originally from Lazio, 6 from Umbria and 4 from Marche regions), representing the 91.96% (1774/1929) of sampled animals. Only one farm (Umbria' s region) was applying a vaccination program against BVDV. The total number of animals sampled was **1,929**, and their age, breed, sex and other characteristics are shown in the ***Table 6***. All animals sampled were Holstein Friesian females. According to the 2 groups of age, 73.25 % (1413/1929) of animals sampled were older than 24 months and 26.74% (516/1929) were younger than 24 months old.

Table 6. Principal characteristics of farms and animals studied in the research

No. Farms	Region	Vaccination	No. of animals	Age [1]		Breed [2]	Sex [3]	Observation
				< 24 m	> 24 m			
10	Lazio	No	821	269	552	HF	F	Monitoring
6	Umbria	No	624	132	492	HF	F	Monitoring
4	Marche	No	329	85	244	HF	F	Monitoring
20			**1774**	**486**	**1288**			
1	Umbria	**Yes**	155	30	125	HF	F	Monitoring
21			**1929**	**516**	**1413**			

[1] m: months; [2] HF: Holstein Friesian; [3] F: Female

4. Serology and Antigen Detection

BVDV ANTIBODY DETECTION. Samples of blood (6 ml) were collected in tubes without anticoagulant and centrifuged at 2500 rpm for 20 minutes in order to obtain sera samples. The sera were refrigerated and tested as soon as possible using a commercial *kit IDEXX HerdCheck ELISA BVDV Antibody Test*. The test was realized following the manufacturer' s instructions. Briefly, the kit requires the sera incubation in antigen-coated wells with BVDV antigen (*NS 2-3 protein*). After 90 minutes of incubation at room temperature, captured BVDV antibodies were detected by anti-bovine horseradish peroxidase conjugate. Unbound conjugate was washed away and substrate/chromogen solution was added. In the presence of enzyme, substrate was converted into a product which reacts with the chromogen to generate a blue color. Upon addition of stop solution, a yellow color was generated. The optical density (OD) of the plate was measured using a spectrophotometer (*Labsystem Multiscan*) at a single wavelength of 450 *nm .*

ANTIGEN DETECTION. Both immunoassay, *Serum/ACE* and *Leukocyte/ACE* are based on the detection of structural glycoprotein E^{rns} of BVDV through the use of monoclonal antibodies (MAbs). In *Serum/ACE*, sera samples were incubated in the plate for 2 hours at 37°C or overnight at 4°C. If in the serum the antigen was present, this was captured on the plate by MAbs. After incubation of the test sample in the well, captured antigen was detected by specific antibodies and horseradish-peroxidase conjugate. Then, unbound conjugate was washed away and substrate/chromogen solution was added. In the presence of enzyme, substrate was converted into a product that reacts with the chromogen to generate a blue color. Upon addition of the stop solution, a yellow color was generated. The optical density (OD) of the plate was measured using a spectrophotometer (*Labsystem Multiscan*) at a single wavelength of 450 *nm .*

In *Leukocyte/ACE*, buffy coat and tissue samples were processed before their use. To obtain buffy coat samples, 5 ml of cold (2-8°C) 0.17M NH_4CL were added in a 10 ml centrifuge tube with 5 ml of EDTA blood, and left for 10 minutes at room temperature. After this step, the tube were filled with cold (2-8°C) distilled water, mixing gently and centrifuged at 1500g for 5 minutes. The supernatant was discharged and an equal quantity of sample diluent included in the kit to the cell pellet was added. After this, the pellet was mixed by vortex and left by 1 hour in room temperature. Finally the tube was centrifuged at 1500 g for 5 minutes and the supernatant was used in the test protocol. In case of tissue samples, 2 grams were cut in small pieces and in a 10 ml tube were added 5 ml of sample diluent (included in the kit), vortexed and left 1-2 hours at room temperature. After this time, the samples were centrifuged at 1500 g for 10 minutes and 50µl of the supernatant were used in the test. Finally, the immunoassay was carried out following the manufacturer' s instructions and using the same principle than *Serum/ACE.*

5. RT-PCR

POOLING SERA SAMPLES. In order to test an economical methodology for the BVDV PI cattle screening, pooled serum samples from all seronegative and young animals was developed. Knowing that serum may have lower amounts of virus compared with other samples, it is recommended to form pools maximum of 10 samples (Muñoz-Zanzi CA., *et al.*, 2000; Ridhpath JF., *et al.*, 2000; Daly R., *et al.*,

2006). Following these recommendations, each pool was formed of 100µl of 5 samples as previously described (Weinstock D., *et al.*, 2001). Some previous experiments have described the detection of BVDV using RT-PCR without RNA extraction in different samples as blood, buffy coat and serum samples, establishing that this step can be omitted without loosing sensitivity of virus RNA detection (Groom J*., et al.*, 1999; Ridhpath JF., *et al.*, 2002; Deregt D., *et al.*, 2002). For that reason we decide to test the RT-PCR using positive controls with both methods (described forward). Pooled samples were tested without previously RNA extraction, using 6 µl of each pool directly to RT (Groom J*., et al.*, 1999). Finally and with the intend to corroborate the validity of PCR, each test was run with a positive control.

VIRUS USED AS POSITIVE CONTROLS. Four BVDV cell-culture infected were used: one was genotype 1 and 3 were genotype 2. The principal characteristics of viruses are present in **Table 7.** The strains were kindly donated by Prof. Carlo Valente (*Laboratorio di Virologia V.Cilli, Università di Perugia) and* Dr. Gian Mario De Mia *(Istituto Zooprofilattico Sperimentale Umbria and Marche, Sezione Perugia*). The cell-culture infected were detached and harvested by the Institution that donate them to us. Briefly, from the cell culture, the medium was aspirated to finally wash the cells with PBS. Then, PBS was aspired and 0.25% of Trypsin was added. Once the cells were detached from the flask, medium was added (containing serum to inactivate the trypsin) and the cells were transferred to a RNAase-free *eppendorf* tube. The total number of cells was unknown. Finally, this material was used for the RNA extraction or was stored at -80°C until their use.

Table 7. *Principal characteristics of virus used in the research*

Virus	Genotype	Year of isolation	Cell culture	Origen	Characteristic
NADL	1	-	BFK [a]	ATCC	Respiratory disorders
TMV-2 (a)	2	1967	MDBK[b]	Nasal swab	Respiratory disorders
TMV-2 (b)	2	1985	BFK [c]	Spleen	Digestive disorders
BVDV-2	2	1970	BFK	Spleen	Digestive disorders

BFK: Bovine Fetal kidney; MDBK: Madin-Darby Bovine Kidney, [a]10 passages; [b] Fourth passage [c] 32 passages and then propagated in MDBK. This strain was originated from TMV-2 (a)

PRIMERS USED FOR THE cDNA AMPLIFICATION AND PCR. Few works have used as a target the sequences of structural and non-structural proteins of the BVDV genome, but the results obtained have shown an important ranges of variability. In other hand, those works that have used as a target the *5' UTR* of the genome, considered as a highly conserved among *Pestivirus*, have shown better results about sensitivity and specificity. Due to the description of the universal *Pestivirus* primers widely used by different authors, their use in different clinical samples and in addition the acceptable results obtained about sensibility, we decide to use the universal primers which amplify a product of 287 pb of the *5' UTR* of the *Pestivirus* genus (Leteiller C. *et al*., 1999). In the same way, primers B3/B4 and B5/B6 were used to amplify and differentiate between genotypes 1 and 2 respectively. Both set of primers amplify a product of 221 pb (**Table 8**).

Table 8. *Primers used to detect Pestivirus genus and for the detection and differentiation of BVDV genotype 1 and 2*

Primer	Sequence	Genome position[a]	Specificity	Size of the amplified product	Reference[b]
BE	5'-CAT GCC CTT AGT AGG ACT AGC- 3'	108-127	Pestivirus	**287 pb** (BE / B2)	1, 3
B2	5' –TCA ACT CCA TGT GCC ATG TAC -3'	395±375	Pestivirus		1,2,3,4,5
B3	5'- GGT AGC AAC AGT GGT GAG- 3'	139±155	BVDV type I	**221 pb** (B3 / B4)	1
B4	5' - GTA GCA ATA CAG TGG GCC - 3'	360±343	BVDV type I		1
B5	5' - ACT AGC GGT AGC AGT GAG - 3'	139±145	BVDV type II	**221 pb** (B5 / B6)	1
B6	5' - CTA GCG GAA TAG CAG GTC - 3'	360±343	BVDV type II		1

[a] According to the NADL genome position
[b] 1) Letellier C., 1999; 2) Letellier C., 2003 3) Groom J. *et al*, 2001 4) Toplak I. *et al*., 2004 5) Luzzago C. *et al*., 2001

Primers were carefully analyzed the nucleotide sequence, the right genome position and the right size of amplified product using the *Genbank* program (http://www.ncbi.nlm.nih.gov/Genbank). Finally, the specificity of each primer was corroborated using the *BLAST program* (http://www.ncbi.nlm.nih.gov/BLAST/), which practically compare similar nucleotide sequences in the same and different organism that we are studying. In this way, the primers chosen were highly specific to the 5' UTR of *Pestivirus* genus, and to the BVDV 1 and 2 genotypes respectively.

RNA EXTRACTION AND cDNA SYNTHESIS. According to different references (Luzzago C. *et al*., 2001; Toplak I., *et al*., 2004), RNA was extracted from 100 and 200µl of each virus-infected cell culture. Due to the high risk of degradation and the low stability of RNA, the RNA extraction was carried out using a commercial kit (*QIAGEN RNeasy Mini Kit*). Briefly, the samples were disrupt and homogenized with buffer RTL (based on Guanidine Thiocyanate). Then, a volume of ethanol at 70% was added and the homogenized was transferred to a Rneasy column included in the kit for being centrifuged to discard the flow-through. After adding RW1 buffer, which create the conditions that promote selective binding of RNA to the membrane, the samples were centrifuged. Two steps of washing with Buffer RPE were realized followed by centrifugation. Finally, RNA was diluted in 30µl of Rnase-free water included in the kit. RNA was used immediately or stored at -80°C until its use.

Using the *QIAGEN Omniscript Riverse Transcription kit,* the cDNA synthesis was carried in 20µl of total reaction, containing 1X of buffer RT, 0.5mM of each dNTP, 1µM of the reverse primer B2 (Vilcek S. *et al*., 1994; Letellier C., *et al*., 1999; Luzzago C. *et al*., 2001 and 2003; Toplak I. *et al*., 2004), 4 Units of Omniscript Reverse-Transcriptase and 1, 2 and 3µl of RNA were tested. In case of RT of positive controls without RNA extraction, 2 µl were used directly from each BVDV cell-infected cultures. In the same way, from sera samples, 6 µl from each pool were used directly without previous RNA extraction (Groom J*., et al*., 1999). The cDNA was synthesized at constant temperature of 37°C in a *Hybaid PCR Express* thermocycler for 60 minutes. Finally, the reverse transcription (RT) reactions were tested immediately by PCR.

cDNA AMPLIFICATION. For the *Pestivirus* cDNA Amplification, we followed the recommendations of a previous research (Leteiller C., *et al*., 1999). In a 50µl of final volume of reaction, the PCR mix contained a final concentration of 1X QIAGEN Master mix (10 mM of tris-HCl, KCl, $(NH_4)_2SO_4$, pH 8.7), 200 µM of each dNTP, 2.5U of QIAGEN *Taq* DNA Polymerase, 1.5µM of each primer (BE/B2) and 3mM of $MgCl_2$. .Different quantities of the RT products (1, 2 and 3 µl) of each viral strain with and witouth previous RNA exctraction step were tested. In case of pooled sera samples, 2 µl of RT were used for the amplification. The amplification program was run with a *Hybaid PCR Express* thermocycler and the amplification program was carried out for 5 minutes at 95°C for an initial denaturation, and then submitted to 35

cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute. Additionally, different annealing temperatures were tested (55, 56, 57, 58, 59, 60°C). A final extension step of 72°C for 7 minutes was used.

In other hand, the PCR to identify and differentiate genotypes was carried out in 50 µl of reaction contained a final concentration of 1X QIAGEN Master mix (10 mM of tris-HCl, KCl, $(NH_4)_2SO_4$, pH 8.7), 200 µM of each dNTP, 2.5 U of QIAGEN *Taq* DNA Polymerase, 75 pM of each primer (B3/B4 and B5/B6 respectively) and 3mM of $MgCl_2$. Different quantities of the RT products (1, 2 and 3 µl) of each viral strain were tested. The amplification program was run with a *Hybaid PCR Express* thermocycler and the amplification program was carried out for 5 minutes at 95°C for an initial denaturation and then submitted to 35 cycles of 94°C for 1 minute, 51°C for 1 minute and 72°C for 1 minute. Additionally, a 55°C annealing temperature was tested. A final extension step of 72°C for 7 minutes was applied. With the aim to reduce the time in each reaction and in order to reduce the costs, a short protocol of time (30 seconds instead of 1minute of each step) and the use of 25µl instead of 50µl of total volume of reaction were tested in both PCRs.

ELECTROPHORESIS. The amplification products were visualized by electrophoresis. Briefly, agarose (*QA-Agarose TM, Qbiogene*) was melted in Buffer TAE 1X (*10X TAE, Shelton Scientific*) in a final concentration of 1.5%. Once that liquid agar was cooled enough, 10µl of etidium bromide (*SIGMA, 10 mg/ml solution*) were added and was poured into prepared casting tray. Ten microlitres of each PCR products were loaded with 2 µl of 1X Bromophenol Blue (*Promega B.B Loading Solution*) into the wells and finally, 5µl of 100 bp Ladder (*RBC100bp Ladder DNA Marker 50 µg/500µl*) were used in each run. The electrophoresis tray (*Scie-Plas*) was filled with Buffer TAE 1X and finally the electrophoresis was run at 100 *V* / 100 *mA* for 40 minutes. Gels were analyzed using a *Gel logic 100 imagining system (KODAK).*

III. RESULTS

1. Antibody and Antigen Detection

The results obtained in the serological survey carried out in Central Italy showed a seroprevalence of **26.43%** (469/1774) in unvaccinated farms, meanwhile **73.56%** (1305/1774) was observed in vaccinated farms. BVDV seroprevalences of each region in unvaccinated farms were: Umbria: 20.35% (127/624), Marche: 24.92% (82/329) and Lazio: 31.66% (260/821) (**Table 9**). In other hand, from the vaccinated farm (Umbria) was obtained a general positivity of 81.29% (126/155), meanwhile 18.70% (29/155) of animals were negative. In the base of group of age, 5.33% (25/469) of animals younger than 24 month old and 85.28% (444/469) older than 24 months old were positive (**Table 10**).

In order to identify PI animals (seronegative / viremic animals), from a total number of 490 animals tested using the *serum/ACE*, a negativity of 100% was observed.

Table 9. General results about BVDV Antibody and Antigen detection in the total number of animals and by region

No. Farms	Region	Vaccination	No. of animals	Antibody BVDV		Serum/ACE	
				+	-	+	-
10	Lazio	No	821	260	561*	0	244
6	Umbria	No	624	127	497*	0	132
4	Marche	No	329	82	247*	0	85
20	**TOTAL**		**1774**	**469**	**1305**	**0**	**461**
1	Umbria	**Yes**	155	126	29	0	29
21	**TOTAL**		**1929**	**595**	**1334**	**0**	**490**

*From all negative animals were selected only animals younger than 2 years old for being tested with serum/ACE

On the basis of serological results, unvaccinated herds were divided in 3 groups: seronegative herds, seropositive herds with no recent cases of BVDV infection and positive herds with recent BVDV infections (**Table 11**). The criteria to differentiate between recent and not recent infection was the consideration of the presence of seropositive animals younger and older than 24 months old respectively (Ferrari G. *et al.*, 1999).

Table 10. Serological and antigen detection results from animals tested according to age.

Region	Vaccination	No. of animals	Antibody BVDV				Serum/ACE	
			< 24 m		> 24 m		< 24 m	
			+	-	+	-	+	-
Lazio	No	821	25	244	235	317	0	244
Umbria	No	624	0	132	127	365	0	132
Marche	No	329	0	85	82	162	0	85
TOTAL		**1774**	**25**	**461**	**444**	**844**	**0**	
Umbria	**Yes**	155	1	29	125	0	0	29
TOTAL		**1929**	**26**	**490**	**569**	**844**	**0**	**490**

2. Modifications of Serum/ACE

Due to the negative results obtained from the identification of PI animals using the *Serum/ACE*, a modification in the incubation time was carried out (Kuhne S. *et al.*, 2005). When overnight protocol at 4°C was used instead of 37°C for 2 hours, no differences were observed in the results. As additional research, 10-fold serial dilutions of TMV-2 (BVDV-2) strain infected-cells culture in BVDV antibody negative serum were tested. Positivity (OD> 0.3) was observed in a 10^{-3} dilution of BVDV strain (**Graphic 1**).

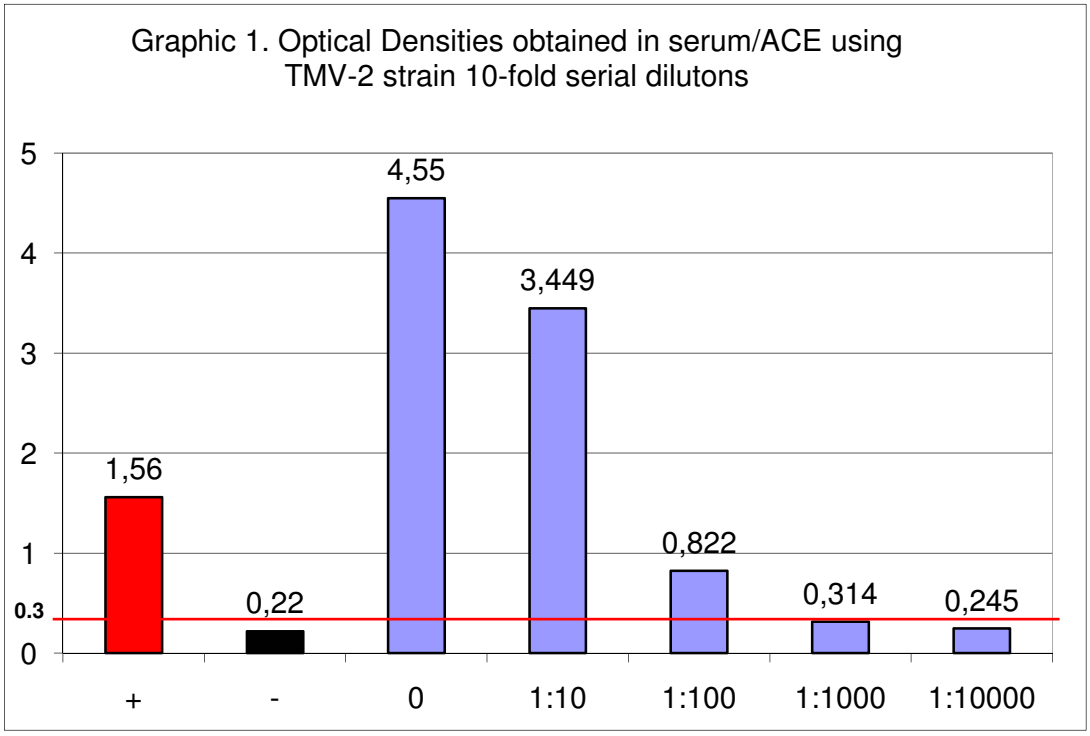


Table 11. Serological and antigen detection results in unvaccinated farms according to the recent and no recent infections classification

Herd status	No. of Herds	No. of animals tested	Ab positive (%)	PI animals
Seronegative herd	**1**(5%)	60	0 (0%)	0
Seropositive herd with no recent BVDV infection	**17** (85%)	1645	324 (19.69%)	0
Seropositive herd with BVDV recent infection	**2** (10%)	224	145 (64.73%)	0
TOTAL	**20**	**1929**	**469 (26.43%)**	**0**

3. RT-PCR

Total RNA extracted from infected cell cultures was submitted to RT-PCR by using the B_EB_2 *Pestivirus*-specific primers, following the author' s recommendations (Leteiller C., *et al.*, 1999). An amplification product of the expected size (287 bp) was amplified from BVDV positive controls strains NADL, TMV-2 (a) and (b), and BVDV-2 (***Fig. 9***). For the RT and cDNA amplification, 2µl of RNA and cDNA of each viral strain showed a better results instead of 1 or 3 µl. The bands with a higher definition were those obtained from the RNA extracted from 100µl than those extracted from 200µl of cell cultures. The expected amplification products were observed in all different annealing temperatures (data not shown). Several unspecific bands were observed in all reactions (***Fig. 9***), even using different annealing temperatures. For this reason, the reduction of final $MgCl_2$ concentration of 1.5mM instead of 3 mM was realized. Finally, all reaction were carried out using the annealing temperature of 57°C as previously recommended. After this modification, a specific band of 287bp was observed without unspecific reactions. A final modifications about " short protocol" and 25µl of final volume were carried out, obtaining optimal results (***Fig.10***). Thus, the optimal RT-PCR protocol was using 2µl of RNA and cDNA of each strain for retrotranscription and amplification, a final $MgCl_2$ concentration of 1.5mM and an annealing temperature of 57°C, using the short protocol in 25µl of final volume.

The cDNAs from the four control strains were amplified with BVDV type I and type II-specific primer pairs B_3B_4 and B_5B_6 respectively (***Fig. 11 and 12***). A fragment of 221 bp was obtained in strain NADL using B_3B_4 primers and from TMV-2(a) and

(b) and BVDV-2 using the B_5B_6 primers, using the annealing temperature recommended of 51°C. No amplification product were obtained using the B_3B_4 primers in genotype 2 strains and B_5B_6 primers in BVDV genotype 1 strains, corroborating their specificity (***Fig. 12***). When we used the annealing temperature of 55°C, unspecific bands were observed, deciding to remain with the original temperature. The final concentration of $MgCl_2$ was 3 mM. Successful results were obtained using the short protocol and 25µl of final volume of the reaction. On the basis of these observations, the optimal RT-PCR protocol was using 2µl of RNA and cDNA of each strain for retrotranscription and amplification, a final $MgCl_2$ concentration of 3mM and an annealing temperature of 51°C, using the short protocol in 25µl of final volume.

In addition, no differences were observed between the amplification products of infected-cell cultures used as positive controls whith and without previous RNA extraction method. In both cases, amplification of the expected band always was observed (data not shown). All sera pooled samples tested with PCR were negative. In each RT-PCR was used a positive control in order to validate the PCR .

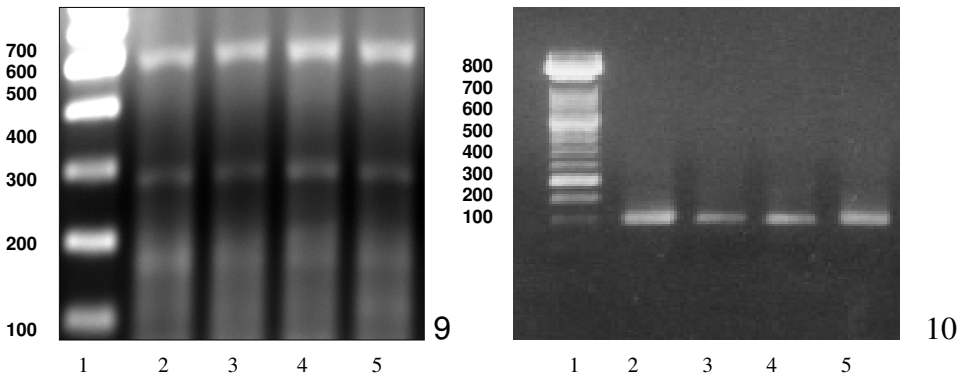


Fig. 9 and 10. Agarose gel 1.5% stained with ethidium bromide. Amplification products of B_EB_2 *Pestivirus* PCR. **Fig. 9**. Lane 1. Ladder 100 bp; Line 2-5: Amplification products of 287 bp of strains: 2)NADL; 3) TMV-2 (a); 4) TMV2 (b); 5) BVDV-2 using a concentration of 3 mM of $MgCl_2$. **Fig. 10**. Amplification products of B_EB_2 *Pestivirus* PCR using short protocol, 1.5mM $MgCl_2$ and 25 µl of total volume. Lane 1: Ladder 100 bp; Line 2-5: Amplification products of 287 bp of strains: 2)NADL; 3) TMV-2 (a); 4) TMV2 (b); 5) BVDV-2.

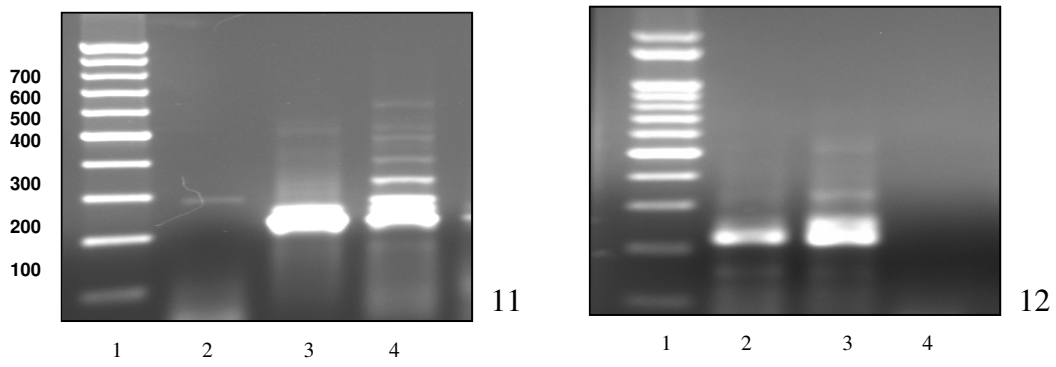


Fig. 11 and 12. Agarose gel 1.5% stained with ethidium bromide. Amplification products of *B_3B_4 (BVDV-1)* and *B_5B_6* (BVDV-2) PCR. **Fig. 11**. Lane 1: Ladder 100 bp; Line 2: Amplification product of *Pestivirus* (287bp) of NADL. Line 3 and 4: amplification products of 221 bp using primers *B_3B_4 (BVDV-1)* in NADL strain with 51°C of annealing temperature; Lane 4: annealing temperature of 55°C. **Fig. 12**. Lane 1: Ladder 100 bp; Lane 2 and 3: Amplification products of B_5B_6 (*BVDV-2*) in strains TMV-2 (a) and BVDV-2 using a concentration of 3mM of $MgCl_2$; Line 4: NADL (*BVDV-1*) strain tested with B_5B_6 (BVDV-2) primers.

IV. SECOND EXPERIMENTAL PHASE

1. INTRODUCTION

From April 2th to May 27 of 2008, Aurora attended a short stage at the Veterinary Diagnostic Laboratory at Rocky Ford (a Branch of the Colorado State University Veterinary Diagnostic Laboratory System), under the supervision of Dr. James Kennedy. The activities that she carried out were:

1. Attendance of multiple seminars in regard to beef cattle health, production, infectious diseases, and their diagnosis at the " Spring 2008 Academy of Veterinary Consultants Conference" ,in Omaha, Nebraska from April 3th to April 5th. From April 7 to April 10, she attended a graduate level course on beef production that included topics on nutrition, economics of production, epidemiology, and record systems. During the same time frame she was afforded the opportunity to tour the United States Department of Agriculture Meat Animal Research Center where over 4,000 beef cows are used for research in genetics and production.

2. From May 7 to May 10, she visited multiple feedlots in the southeast part of Colorado in collaboration with Pfizer Laboratories, with the objective to learn and understand several practices about management and production in beef cows. Additionally, on May 22, she participated in the ear notches sampling procedure in a feedlot with a previous BVD history. The following day, Aurora observed and participated in the gathering and processing of over 300 calves in preparation for summer grazing.

3. The rest of the time, she observed and participated in several activities at the Veterinary Diagnostic Laboratory at Rocky Ford. Those activities included Bovine Viral Diarrhea (BVD) diagnosis, components of a Colorado' s Voluntary BVD Control Program, the organization and pooling procedure of ear notches samples, RNA extraction, BVD viral detection using a specific Polymerase Chain Reaction (PCR), and the use of Antigen Capture ELISA on ear-notches surnatants to detect BVD virus.

VETERINARY DIAGNOSTIC LABORATORY
COLORADO STATE UNIVERSITY
Colorado Voluntary BVD Control program

The Diagnostic Laboratory at Rocky Ford has implemented a screening test to detect BVDV PI animals **(Fig. 13).** They have been used it for over two years and it

appears to have great potential as a herd screening tool. Five other States of America are also implementing similar efforts and the laboratories in those States are preparing to offer this type of test. Until current days, the Rocky Ford Laboratory had run over 40,000 samples on this system.

To achieve the objective, ear notches samples are used. The skin must be placed in a red top tube, and if not submitted fresh, it should be frozen until ready to submit. A small amount of a buffered solution is placed in the tube to immerse the skin sample, and then, an amount of the fluid is removed and combined with other samples up to a maximum of 100 samples. A PCR test is performed on this combined sample for BVD along with a positive control, in order to discard any kind of inhibition. The fee is $50 per pooled test plus other small fees, so if all the samples are negative, the test would only cost $0.61 per head. However, if the test is positive, further testing is needed to identify the PI animals. The test used for this propose is the ACE and it costs about $5 per sample.

Statistical evaluation of RT-PCR to detect BVDV in pooled samples has shown a sensitivity and specificity of 100% and 97.35% respectively. The correct classification rate has been described of 97.7% with a Predictive Value of Positive and Negative Test of 85.1% and 100% respectively. This same test, has shown a correlation of 100% with IHC and ACE tests (Kennedy JA., 2006 a and 2006b).

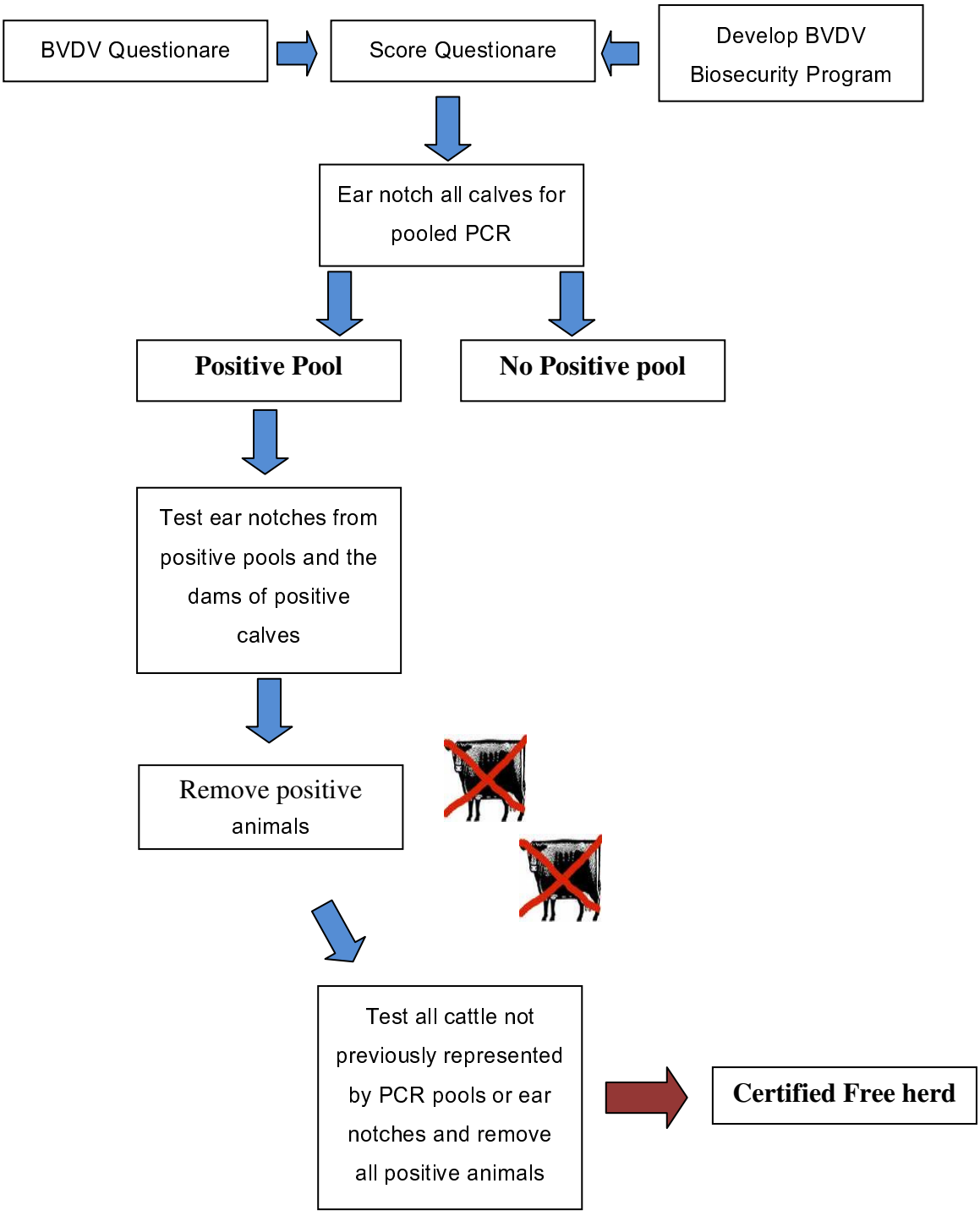
In order to collect the information from each farm, the farmers must submit a questionnaire to the Laboraratory prior to start the program. Information as pregnancy and weaning rates, mortality in calves, vaccination, percentage of abortions, open days, previous history of BVDV, movement / introduction of animals, presence of respiratory disease and knowledge about BVDV are required (http://www.dlab.colostate.edu/BVDControlProgram/bvdcontrolprog_main.cfm).

2. METHODOLOGY

EAR NOTCHES SAMPLES.Using a commercial ear-notcher (*Agrihealth Ear Notcher Pattern A V-shape*), triangular ear notches (~2 cm) were obtained from a clean portion of the ear (**Fig 14 and 15**). It was important to disinfect the ear-notcher before collecting next sample using a 10% bleach solution and rinsed with fresh water to remove the bleach.The procedure was carried out wearing gloves. Carefully, each sample was taken with a clean and disinfected clamp to be collected into a sterile red top tube without touching it during the management (**Fig 16**).

Samples were sent immediately to the Laboratory using a cold package for the transportation.

Fig. 13. BVDV Control Program Flowchart



POOLING SAMPLES PROCEDURE. The work area was wiped down with *ELIMINase* prior starting, between each pool and when finished. Red top tubes with ear notches were arranged in 100-tube box and then the caps were removed. A *LABmax* dispenser was used to add 1 ml of PBS 1X to each tube, and then the

boxes were covered using a plastic bag to let them stand for at least 10 minutes at 4°C. After 10 minutes of incubation, samples were vortexed and using a sterile graduated transfer pipette, 250µl of ear notch fluid from each sample tube were recollected into a 50 ml *Falcon tube* labeled to form a pool (**Fig. 17**). Pool size never was greater than 100 samples. When pooling was completed, *Falcon* tubes were mixed by inversion gently and refrigerated at 4°C for at least 1 hour before RNA extraction.

RNA EXTRACTION OF POOLS. Using the disposable pipettes, 350µl of each pool sample were collected for the RNA extraction. This procedure was carried out using the commercial kit *QIAamp RNA Blood Mini Kit* following the manufacturer' s instructions. Briefly, the samples were disrupt and homogenized with buffer RTL. The lysate (RTL/sample mixture) was pipette into a purple *QIAshredder Mini Spin Column* and centrifuged to discard the flow-through. Then, a volume of ethanol at 70% was added and the homogenized was transferred to a labeled *QIAamp Mini Spin Column* included in the kit for being centrifuged to discard the flow-through. After adding RW1 buffer, which create the conditions that promote selective binding of RNA to the membrane, the samples were centrifuged again. Two steps of washing with Buffer RPE were realized followed by centrifugation. Finally, RNA was diluted in 30µl of Rnase-free water included in the kit and was used immediately.

PRIMERS.The selected primers **SBA-F***5-GTAGTCGTCAGTGGTTCG-3* and **SBA-R** *5-GCCATGTACAGCAGAGAT-3* were used to amplify a product of 145 bp of the high conserved 5' UTR of the BVDV 1 and 2 genotypes(Ridpath JF., *et al*., 1994).

cDNA SYNTHESIS AND PCR. In order to detect PCR inhibitions, a duplicate template for each sample was spiked with BVDV viral RNA for a final concentration of at least 10 and no more than 100 viral particles per ml. The BVDV positive control should be at a concentration of 1000 to 10,000 viral particles per ml. At the same time, a positive control was always run in each PCR reaction.

For the cDNA synthesis, the first step was to prepare the template mix formed by 4.75µl of RNA and 0.5µl of each primer (*Promega*, 50µM). The mix was heated at

70°C for 10 minutes in the thermocycler (*Techne TC-412 Thermal Cycler*) and then cooled on ice for 10 minutes. Then, the samples were placed in a frozen rack in a -70°C freezer for 10 or 15 minutes. The second step was to prepare 4.75 µl / sample of RT-Mix adding 2µl of 5X buffer (*Promega, 250 mM KCl, 250mM Tris HCl Ph 8.3, 50 mM* $MgCl_2$), 1µl dNTPs (10 mM/each), RNAsin 0.25µl (*Promega, 10 Units*), 5U of AMV RT(0.5µl) (*Promega Reverse Transcriptase*) and 1µl of NaPP. Once prepared, 4.75µl of RT-Mix were added to each frozen template (4.75µl), pipetting to the bottom of the tube through the mineral oil and expelling next to ice cube. Samples were incubated at 45°C for 1 hour using the thermocycler.

To prepare PCR Master Mix, in a total volume of 35µl were added 21µl of water, 4.5µl of 10X Buffer, 1.5µl of $MgCl_2$ (50mM), 1µl of dNTPs, 1µl of each primer *SBA/B-F* and 5 µl of 1% Triton-X. This mix was added to each reaction tube and put in the thermocycler to denature at 99°C for 4 minutes. After this incubation, tubes were hold in thermocycler at 90°C while 5µl of Taq (*Bioline Taq Polymerase*) was added to each tube, obtaining a total volume of 50µl. Finally the amplification program was run at 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. A final extension step of 72°C for 5 minutes was carried out.

ELECTROFORESIS. The amplification products were visualized by electrophoresis. For that propose, 5 gm of agarose (*GenePure LE Agarose*) were melted in 140 ml of 0.5X TBE buffer (*5X TBE Buffer*). Once that liquid agar was cooled enough, 15µl of Etidium Bromide (1*0 mg/ml solution*) were added and was poured carefully into prepared casting tray. A pipette tip was used to move any bubbles away from the teeth of the combs to the edge of tray. Twenty microlitres of each PCR product were mixed with 1µl of the Loading Dye Solution (*Promega Bromophenol Blue Loading Solution*) into the wells, and 5µl of 100 bp Ladder (*New England BioLabs 100bp DNA Ladder*) were used in each test. The electrophoresis tray was filled with 0.5X TBE Buffer. The electrophoresis was run at 80 *V* for 1 hour and gels were analyzed using a transilluminator.

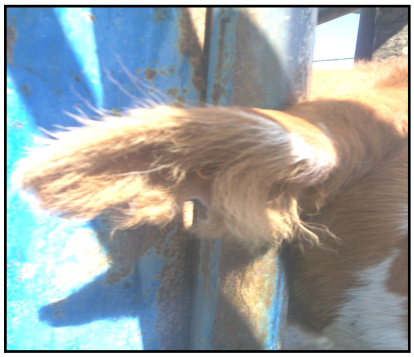


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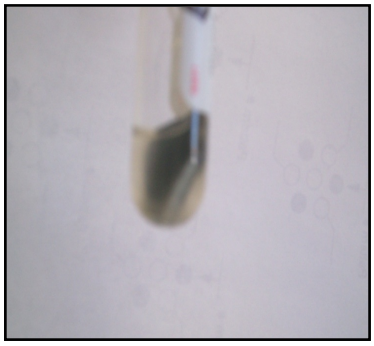
Fig. 14 and 15. (Left) Ear from a PI animal. (Right) Ear notcher and clamps used for sampling procedure.



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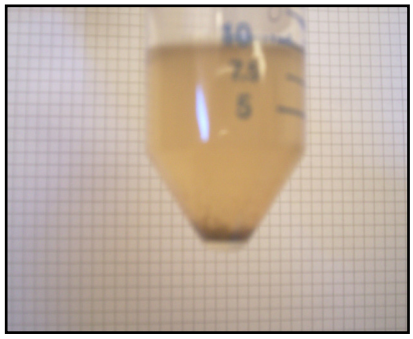

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Fig. 16 and 17. (Left) Ear notch collected in a sterile red top tube. (Right) Pooled samples from 50 ear notches surnatants.

INTERPRETATION OF PCR RESULTS. Ladder and BVDV positive control bands were used to classify the tested pools bands as " BVDV not detected" or as " Positive" if a band of 145 bp was observed. A positive BVDV band always had to be present in spiked samples. If not, a result of " PCR inhibition" was given. There were three possible outcomes for PCR results. " BVDV not detected" is self-explanatory. A result stating " Positive" means that virus was identified in the pool but may be the result of the presence of a PI animal or in some occasions, an acutely infected animal (less than 2% of pools have been identified and no positive animals found). The third possible outcome was " PCR inhibition" and this result occurred when some foreign substance contaminates the sample (less than 4% of pools show inhibition). Whenever the virus is detected or when pools show inhibition, individual samples are automatically evaluated.

BVDV ANTIGEN CAPTURE ELISA TEST

The commercial test *BVDV Antigen Test Kit (HerdCheck IDEXX, USA)* was used to test individually the ear notches surnatants from positive pools. The kit is based on the detection of structural glycoprotein E^{rns} of BVDV through the use of monoclonal antibodies (MAbs) and was carried out following the manufacturer' s instructions. Briefly, micro-wells were washed with washing solution before starting. Ear-notches surnatants were incubated at room temperature (RT) for one hour and after were washed away to eliminate the unbound material. A working detector reagent was added and incubated for 1 hour at RT and washed away. After this incubation, captured antigen was detected by specific antibodies and horseradish-peroxidase conjugate. Then, unbound conjugate was washed away and substrate/chromogen solution was added. In the presence of enzyme, substrate is converted into a product that reacts with the chromogen to generate a blue color. Upon addition of the stop solution, a yellow color is generated. The optical density (OD) of the plate was measured using a spectrophotometer (*Emax Miroplate Reader)* at a single wavelength of 450 *nm .*

An OD <0.2 was catalogued as negative, between 0.2-0.39 as suspect and >0.39 as positive. A positive BVDV ACE test may not be indicative of PI animals. A positive result on a second test at least 3 weeks after the initial sample can confirm a PI animal. Suspected animals resulting for the ACE were retested twice, first using the standard working detector regent (monoclonal goat anti-BVDV antibody) and then using a " modified" working detector (containing no detector antibody) with the aim to obtain a ratio between optical densities.

3. GENERAL RESULTS

In a period from April 5th to May 26 of 2008, a total number of 14,508 ear notches samples were received in the Laboratory. The samples were originally from Colorado state, but also from others states of America as Nebraska and New Mexico. From these samples, a total of 360 pools of an average number of 40 samples were tested. From these pools, 28 were positive to RT-PCR and when samples were tested individually using the ACE, 15 animals were catalogued as positives. All positive animals in ACE were retested 3 weeks later and confirmed as PI animals. Positive animals were Hereford breeding with an average age of 4 months.

V. DISCUSSION AND CONCLUSIONS

In Italy, little research has been done concerning about BVDV seroprevalence and at this time no compulsory/voluntary control program exists in order to detect and eliminate PI animals. For that reason, the study of seroprevalence in Central Italy was an important research to understand the BVDV epidemiology in this area. The serological investigation was realized using a commercial ELISA that has shown a high sensitivity and specificity of 99.5% under field infections. Also it has demonstrated a strong association of 96.3% with the Virus Neutralization Test, which is considered as the golden standard of the serology in BVDV infections (Solis-Calderon JJ. *et al.*, 2005). The serological survey carried out in unvaccinated herds in Central Italy showed a relative BVDV seroprevalence of 26.43%, meanwhile the seroprevalence obtained by regions were 20.35%, 24.92% and 31.66% in Umbria, Marche and Lazio respectively. These results demonstrate the presence of BVDV infection in a low prevalence and in general reveal that the situation in Central Italy is not critical about BVDV compared with others regions or countries, where BVDV is endemic reaching seroprevalences up to 90% (Greiser-Wilke I., *et al.*, 2003; Moennig V., *et al.*, 2006; Lindberg A., *et al.*, 2005).

In a previous survey on bulk milk samples of 379 dairy herds in the Umbria region in Central Italy, antibodies were demonstrated in 45 samples, resulting in a prevalence at herd level of 11.8% (Luzzago C.,*et al.*, 2001). In other research, a seroprevalence of 53.3% in 29 unvaccinated farms in northern Italy using the serum neutralising test has been reported (Luzzago C. *et al.*,1999). In other hand, a seroprevalence at individual level of 31.4% was reported in Rome province (Ferrari G. *et al.*, 1999) using a competitive ELISA test. Only one research has shown a pick of seroprevalence of 91% in northern Italy during a period of ten years, but the study did not differentiate between non-vaccinated and vaccinated herds (Cavirani S. *et al.*, 1992).

In other hand, it is important to refer that in Italy there are important differences in density, size and management between North Italian and Central Italian herds. In general, North Italian herds are more numerous and the animal-to-animal contact is closer, resulting in higher risk of viral transmission and consequently in higher seroprevalences (Lindberg A. and Houe H., 2005; Ezzano P., *et al.*,2008). It was shown that the herds with high cattle population density had higher prevalence of infection than the herds which were smaller (Houe H., *et al.*, 2006; Hult L., *et al.*, 2005). For that reason, the seroprevalences obtained in Central Italy regions could

be lower than those observed in other regions due to the different herd size and management system.

The results obtained about seroprevalence acording to the group of age showed the tendency of a higher risk among older (>2 years old) animals compared to younger (aged <2 years) animals. This phenomena has been previously reported (Mockeliū niene V., *et al*., 2004; Garoussi MT., *et al*., 2008) and it has been demonstrated that animal age has an important influence on the prevalence of BVDV infection. Some reports have shown that in the third and consecutive years of life, the number of seropositive animals increases reaching its maximum in the age groups of 5 and 7 years. The increase in antibody prevalence by increasing age is probably due to the higher exposition to the antigens and that BVDV antibodies in most cases are lifelong (Mockeliū niene V., *et al*., 2004).

On the basis of serological screening, herds were classified with and without recent cases of infection. Five percent of farms were seronegative, 85% were seropositive with no recent BVDV infection and only 10% had a recent or on-going infection. Previous researches carried out in different parts of Italy have shown that the seroprevalence in the presence of recent infections within farms involved and in the absence of a systematic control program were of 8.8% (Ferrari G. *et al*., 1999) and 33.8% (Luzzago C. *et al*., 2008) respectively. In this way, we can consider that the percentage of recent infected farms observed in our research is similar to that reported in herds that are participating in a systematic control, where PI animlas are constantly eliminated. The possibility of external sources of infection in seropositive farms with recent infections must be considered.

In other hand, in vaccinated herds was obtained 73.56% of positivity due to the post-vaccination immune response mounted by animals, meanwhile the general negative percentage was 26.44%. It is possible that the reason why some animals are not developing a post-vaccination immune response could be for the failure in vaccine application and/or conservation (4°C), mistakes in animal' s control during the vaccination (missed animals during vaccination), an incomplete protection against BVDV infection due to immunosuppression for the possible presence of other infectious agents as Bovine Immunodeficiency Virus (Zhang S*., et al*., 1997) and the use of inactivated vaccines that generally produce a weaker last-long immune response (Potgieter LN., 1995; Platt R., *et al*., 2008), adding to this situation the possible lack or failure in booster vaccination.

In Italy, the presence of BVDV PI animals has been reported previously, principally in the northern part of the country (Falcone E., *et al*., 2001; Piccinini R., *et al*., 2006; Luzzago C., *et al*., 1999, 2001 and 2006), where the presence of an unusual number of PI animals suffering MD in one of more farms has been reported, phylogenetically related to BVDV genotype 1 (Ciulli S., *et al*., 2008). One of the principal objectives of this research was to identify PI animals within sampled farms in order to evaluate the use of different diagnostic tools for their identification. For that propose, young animals (<2 years old) were tested, based on previously reports that show that PI cattle die during the first years of life (Tautz N.,*et a*l., 1998; Potgieter LN., 1995; Smith DR. *et al*., 2004).

In our study, from 490 young animals tested, a negativity of 100% was obtained in BVDV antigen detection, even if overnight incubation method was used with the intent to improve the sensitivity of the test (Kuhne S. *et al*., 2005). Technical causes have been excluded when 10-fold serial dilutions of *BVDV TMV-2* strain were tested with optimal results, thus it was possible to corroborate that viremic and PI animals were not detected. For the antigen detection only serum samples were used, and although some authors recommend its use to detect the BVDV antigen with good results compared with other tests (Saliki JT., *et al*., 2000; Kuhne S. *et al*., 2005; Kampa J. *et al*., 2007; Hill FI., *et al*., 2007), other authors express their doubts about the ability of the test on sera samples to detect PI animals (Grooms DL. *et a*l., 2002; Gripshover EM., *et al*., 2007). However, test has been validated by the manufacturer for using sera samples, adding that are easy to collect and are most frequently sent to the laboratories for BVDV diagnosis.

The *Serum/ACE* used for this research is based on monoclonal antibodies to capture the E^{rns} BVDV antigen, which is thought to be highly conserved among BVDV strains (Bolin SR., *et al*., 2004; Vilcek S., *et al*., 2005). According to the manufacturer, this test has shown a sensitivity and specificity of 91% and 97% respectively in field infections. Application in control programs of the Serum/ACE for the detection of PI animals has shown good results and agreement with other commercial kits as well as IHC, RT-PCR and indirect immunoperoxidase tests (Cornish TE., *et al*., 2005; Kennedy JA., *et al*., 2006 a and 2006b; Hilbe M., *et al*., 2007). *Serum/ACE* is used successfully in countries that have started a BVDV control programm as Netherlands, where blood pooled samples are tested first by RT-PCR and from the positive pools, the animals are tested individually with the kit

(Mars MH., *et al*., 2005). Controversially, Gripshover EM., *et al*, (2007) demonstrated that there is a unique mutation in the portion of the genome coding for the E^{rns} glycoprotein envelope associated with failure of IHC and ACE assays to detect some field strains previously detected by virus isolation and PCR testing from PI animals.

Concerning about the RT-PCR standardized, it was considered as specific to the high conserved region of the 5' UTR, and have been used by other authors with high sensitivity and specificity (Leteiller C., *et al*., 1999 and 2003; Groom J. *et al*., 2001; Luzzago C. *et al*., 2001;Toplak I. *et al*., 2004). The fact that RNA from all cell-infected cultures tested showed a better integrity using 100µl of initial material than 200µl could be due for the high quantity of RNA of the samples which exceeds the capacity of RNA binding to the Rneasy membrane, reducing the RNA yields. About the PCR to detect *Pestivirus*, the $MgCl_2$ concentration of 3mM had an important influence in unspecific reactions and when the final concentration of 1.5 mM was used, it was possible to eliminate the unspecific bands. The expected amplification products were obtained from all different strains. About the PCR to identify and differentiate between BVDV genotypes, no cross-reactivity was detected between type-specific primers. In both PCRs, the use of short protocol and the reduction of 25 µl instead of 50µl of the total volume considerably reduced the cost and the time to carry out each reaction.

No differences were observed between the amplification products of BVDV infected-cell cultures used as positive controls with and without previous RNA extraction method. This phenomena has been described before, using samples as blood, buffy coat and serum. These results consent to conclude that RNA extraction can be omitted without loosing sensitivity of virus RNA detection (Groom J*., et al*., 1999; Ridhpath JF., *et al*., 2002; Deregt D., *et al*., 2002), and decreasing at the same time the economical costs of the diagnosis.

With the aim to develop an economical diagnostic test for the screening PI cattle, a RT-PCR assay in pooled sera samples was used in this research. It is important to consider the optimal size for pooled sera lots that depends on herd size and the ages of the cows to be tested. Sera from older multiparous cows with a lower prevalence of PI can be pooled into larger lots of sera samples than sera from animals of 2 years old or less, which have a higher expected prevalence of BVDV infection (Weinstock D., *et al*, 2001). For this reason, in this research only animals younger than 2 years old were tested in order to increase the possibility to detect PI

cattle. Some works have reported that serum may have lower amounts of virus compared with other samples as blood or buffy coat, recommending that pools formed must to be maximum of 10 samples (Muñoz-Zanzi CA., *et al*., 2000; Daly R., *et al*., 2006). Following this recommendations, sera pools for this research were formed by 5 samples in order to avoid the decrease of sensitivity using RT-PCR.

Previous reports have shown that a single PI animal can be detected in pools of 30 (Smith RL.,*et al*., 2008) and until 100 sera samples (Weinstock D., *et al*., 2001). Additionally, high antibody titers in pooled sera samples had no appreciable effect on the sensitivity of detection of BVDV by RT-PCR and the rate of degradation of the viral RNA and the stability of BVDV in serum depends on the initial virus titer, sample handling, and the purity of the serum (Weinstock D., *et al*., 2001). These studies estimate the diagnostic sensitivity of RT-PCR for BVDV and confirms that it is a useful diagnostic tool for sera pools screenings. Our results have showed that all tested pools were negative using a highly sensitive RT-PCR (Luzzago C. *et al*., 2001;Toplak I. *et al*., 2004), presuming that the results obtained were truly negative.

Several factors must be considered concerning about the negative results. One of the most important is the low prevalence of PI animals, ranging from 0.5 to 2% (Brock KV., 2003; Greiser-Wilke I., *et al*., 2003; Niskanen R., *et al*., 2003; Moennig V., *et al*., 2006). In other hand, It has been observed that a low percentage of PI animals do not present detectable titers of viremia due to the antibody production against some homologous BVD virus spontaneously mutated, representing the " incomplete immunotolerance" theory (Fray MD., *et al*., 2000). In the same way, some researches have reported the intermittence and variation of viremia simultaneously with its declination with the time, specifically after 2 years old of age (Brook KV., *et al.,* 1998; Fray MD., *et al*., 1998 and 2000; Baule C., *et al*., 2001; Grooms DL., *et a*l., 2001). These phenomena could explain some missing periods of viremia that could be important contributors in the inefficient detection of PI animals.

PI animals are considered the principal source of infection in a farm and within herds, and direct contact between these animals and susceptible ones is the most important route of infection (Houe H., 1999; Greiser-Wilke I. *et al*., 2003; Smith DR., *et al*., 2004; Lindberg A., *et al*., 2005). In this work, the presence of BVDV infection has been corroborated in a low percentage in the absence of PI animals. In addition, 10% (2/20) of the total farms samples are presenting on-going infections. Some works have reported the transmission of the disease within herds even if the

absence of PI cattle, guessing the important role of the transient infected (TI) animals that can spread the virus for long periods of time (Moerman A. *et al*., 1993; Moen A. *et al*., 2005;). In the same way, a case of severe course of a primary BVD infection in a seronegative herd after the introduction of a transiently infected (TI) calf was described (Sol J. *et al.*, 1989).The probability of horizontal transmission during a contact with a TI animal stands in sharp contrast to that of PI animals, due to the intermittent shed of relatively low amounts of virus. In fact, there are experimental results that indicate that the primary infection may be only rarely propagated (Niskanen R., *et al*., 2003). Even this feature, the probability of vertical spread resulting from a TI animal is much higher than the probability of horizontal spread. In fact, most new PI detected in an infected herd will be the result of transient infections in dams with a normal immune response (Lindberg A. and Houe H., 2005).

Nevertheless, external routes of infection must be considered as important ways of transmission, as the use of contaminated semen and embryos (Perry GH., 2007; Gard JA., *et al*., 2007), contaminated vaccines (Falcone E., *et al*., 1999; Barkema HW., *et al*., 2001), unhygienic treatment procedures and contaminated housing conditions (Niskanen R., *et al.*, 2003; Ezzano P., *et al*., 2008) as well as airborne transmissions between animals (Mars MH., *et al*., 1999; Smith DR., *et al*., 2004).

In other hand, it has been shown that the prevalence of seropositive animals in unvaccinated herds with one or more PI animals generally is high, ranging from 87% to 98% (Waldner CL., *et al*., 2005; Seki Y., *et al*., 2006;Houe H., *et al*., 2006), contrary in those without PI animals ranging from 8 to 29% (Seki Y., *et al*., 2006) and reaching until 49% (Garoussi MT., *et al*., 2008). Therefore, a high prevalence of seropositive animals is an indirect evidence of the presence of PI animals. This connection has been utilized as a means of making a preliminary herd diagnosis by testing a small herd sample for antibodies against BVDV in areas where BVDV vaccination is not available. In areas where vaccines are used, the problem is more complex, because it has to be determined whether the antibody titer is due to vaccination or due to exposure to a PI animal, but generally, the antibody titers in herds with PI animals are higher than those among cattle in herds without PI animals, even if the cattle in the herds had been vaccinated with a killed vaccine (Ståhl K., *et al*., 2008). However, some herds without recent BVDV exposure may have older animals with high titers, presumably resulting from a previous exposure

to a PI animal (Houe H., 1995; Ståhl K, *et al*., 2007). The seroprevalences obtained in this work were considered as low, and we can presume that are similar than those reported in BVDV seropositive farms without the presence of PI animals.

Different surveys show considerable variation in the prevalence of PI animals as well as prevalence of antibody positive animals. Several differences in cattle population structure, cattle density, herd size, housing systems, animal movement / introduction / purchase, bio-security practices and management in general could be important risk factors playing an important role in transmission and persistence of BVDV (Valle PS., *et al*., 1999; Wittum TE., *et al*., 2001; Sthal K., *et al*., 2007). For that reason, the low herd density in the central regions of Italy and the probable right management practices within herds must not be excluded as contributors and possible factors that could reduce the presence of PI animals.

Finally, the use of contemporary sampling of different materials than serum in the same animal (Cornish TE. *et a*l., 2005; Edmondson MA., *et al*., 2007;Hilbe M., *et al*., 2007b), a serum re-sampling protocol with a difference of at last 30 days between samples (Grooms DL. *et al*., 2001 and 2002), the examination of white blood cells (Baule C. *et al*., 2001; Gogorza LM. *et al*., 2005) and ear notch samples (Kuhne S. *et al*., 2005) are practices that could increase the possibility to detect BVDV PI animals. The serum/ACE has shown a better results when ear notch samples are used (Kennedy JA., *et al*., 2006 a and b; Hill FI., *et al*., 2007),meanwhile the examination of white blood cells (buffy coat) with the ACE test is also recommended to detect BVDV antigen with a higher sensitivity (Baule C., *et al*., 2001; Cornish TE., *et a*l., 2005; Gogorza LM., *et al*., 2005).

Recently, no compulsory BVDV control Program exists in Italy.In geographical areas where governments and organisations of farmers do not consider BVDV to be a priority, control is dependent on voluntary measures. In Italy, the herd seroprevalence of BVDV ranges from 57% to 95% (Ferrari G. *et al*., 1999; Luzzago C., et al., 2008) and there is high BVDV genetic variability relative to other EU countries (Luzzago C.,*et al*., 2001). Despite this,a temporary compulsory program in Italy has been developed in the North-Eastern region (Luzzago C., *et al*., 2008) and in a voluntary way in Rome province for a short period of time (Ferrari G., *et al.,* 1999). Both programs were based on the identification and elimination of PI animals. According to our results, no PI were identified in the Central regions sampled, even the presence of BVDV infection. For that reason, and in the absence of national or

local controls, there is a need to quantify the risk of introduction and spread of BVDV in order to increase the awareness of farmers in order to spread the right knowledge to prevent new infections that could increase the prevalence. At the same time, we strongly recommend the application of other diagnostic techniques such as IHC, RT-PCR and *ACE* using buffy coat and/or ear notches samples to detect the presence of PI animals (Cornish TE. *et a*l., 2005; Hilbe M., *et al*., 2007; Hill FI., *et al*., 2007).

BVDV is a common disease of United States cattle herds (Larson RL., *et al*., 2004 and 2005; Loneragan GH., *et al*., 2005). The disease affects producers and the livestock industry through increased treatment expenses, calf and pregnancy losses, and decreased weight gain (Gunn GJ., *et al*., 2004). During the short stage at the Veterinary Diagnostic Laboratory of the Colorado State University, a total number of 14,508 ear notches samples were submitted to the Laboratory for testing for BVDV PI animals. All samples were obtained from farmers that were agree to participate in the voluntary program. Following the protocol of testing individually the samples derived from positive pools with *ACE,* a prevalence of 0.1% of PI was obtained. This prevalence has been described everywhere, ranging from 0.5 to 2% (Brock KV., 2003 and 2006; Moennig V., *et al*., 2005a; Lindberg A., *et al*., 2006).Current practices in USA cattle industry do not limit the risk of exposure to PI animals. A recent feedlot study showed that 30.8% of 240 pens would contain at least 1 PI animal, given a prevalence of 0.4% PI animals and a total of 21,743 head (Ridpath JF., *et al*., 2006b).

The dairy industry has used RT-PCR to successfully detect the presence of BVDV in bulk-tank milk samples, but that particular source is not available in beef cow operations. Screening ear notches by pooled RT-PCR could offer a low-cost and highly sensitive test to detect BVDV (Kennedy JA. *et al*., 2006 a and b). Screening ear notches with pooled RT-PCR followed by AC-ELISA with PBS solution to detect individual positive samples offers the potential for performing serial testing to minimize expense and laboratory time and could allow potential PI animals to be removed from the herd in less than 72 hours of laboratory time at a reasonable cost to the producer. In addition, RT-PCR used in this laboratory has shown to be highly sensitive, reaching a 100% on pooled samples when at least 1 positive animal to *ACE* is contained in a pool of 100 samples. Therefore this system of 100 animals provided a screening method that detect all the individual positive IHC and positive *ACE* samples (Muñoz-Zanzi CA., *et al*., 2000; Kennedy JA., *et al*., 2006a and b).

Increasing the pool size above 100 might result in a failure to detect the virus if the dilution were to become great enough. Conversely, decreasing the pool size could allow for more frequent detection of TI animals and a subsequent decrease in the number of times the pool was confirmed to contain a positive *ACE*.

Important and different results were observed about the prevalence of PI animals between surveys carried out in Central Italy and in Colorado (USA), being 0% and 0.1% respectively.Those differences could be due to the different number of animals sampled. This difference was important because in Colorado the voluntary program is well organized, the farmers are agree to participate and eliminate infected animals and the most important is that they are conscious about the importance and risk that BVDV means for their herds, so they participate constantly in the program. In other hand, in Italy there is no compulsory/voluntary program developed in the Central area, becoming difficult to convince the farmers to get involved.

Another important point to discuss is the different samples used in each survey (serum vs. ear notch). In previous researches, using ear notches samples to detect PI animals with the *ACE*, it has been possible to identify until 100% of animals previously detected by VI and RT-PCR (Edmondson MA., *et al*., 2007; Hill FI., *et al*., 2007). Both sera and ear tissue samples from PI calves have been tested and while sera samples were negative in ACE after intake of colostrum, the ear tissue samples could be detected positive for BVDV all the time. In the same way, testing multiple samples derived from the same ear from PI cattle yielded positive results and low variation (Kuhne S., *et al*., 2005). Contrary, sera samples used for BVDV antigen detection have shown a wide variability in the results (Saliki JT., *et al*., 2000; Plavsic MZ., *et al*., 2001; Kuhne S., *et al*., 2005).

Differences between management and herd practices are factors that must be considered. In beef herds, PI suckling calves are commonly in contact with the breeding herd during early gestation when the foetus is susceptible to BVDV persistent infection. As a result, PI suckling calves are considered to be the primary source infection and maintenance of BVDV within breeding herds. Efforts to eradicate PI animals in infected beef herds should therefore emphasize testing of calves prior to the beginning of the breeding season, with follow-up testing of dams of positive calves and animals without calves available for testing. The opposite situation could be observed in dairy cattle, where the calf-weaning is carry out

immediately or some hours after calving, decreasing the possible contact between newborn PI animals and dams (Talafha AQ., *et al*., 2008; Ezanno P., *et al,* 2008).

The importance and the role that wild animals as deer in the BVDV epidemiology in Colorado state must be considered. Previous researches have reported that even that the prevalence of BVDV PI in deers is low, some percentage of adult animals could be playing an important role in the transmission and maintenance of BVDV to bovine herds (Duncan C., *et al*., 2008).

Epidemiological investigations have shown that demographic factors such as herd size and herd density are significant predictors for the prevalence of infection in populations where BVDV is endemic (Valle PS., *et al*., 1999; Houe H.,1995 and 2003; Brock KV., 2004b). It has been estimated that in an endemic area the maximum possible number of PI animals will be around 2% (Greiser-Wilke I., *et al*., 2003). However, in a few regions, the infection is present at a much lower prevalence. Studies from the US indicate that BVD virus is present in relatively few herds, but occasionally in high numbers in these herds, revealing several herds with multiple BVDV positive animals (Houe H., 2003). In America, the use of having animals on pasture as well as herd size and purchase intensity, are considered important risk factors significantly associated with presence of PI animals in the herds. Contrary, In Central Italy, the current herd management practices and low cattle density may be contributors to the low presence of PI animals. Thus, it seems that there are several and important epidemiological differences between both studied regions.

In conclusion, BVDV infection in bovine herds of Central Italy is not critical, but BVDV is present in all the farms included in the study. The absence of PI animals suggest that other routes of transmission need to be considred and investigated. A voluntary eradication program in Italy could improve the health status of the animals and reduce the economic losses. A diagnostic system like that one existent in Colorado could be easily developed, but the main problem to be solved is the consensus and participation of the farmers. Dissemination of information should be done at national level in order to explain to the farmers the advantages that they would have in eradicating BVDV infection in their farms and in order to involve them in the eradication program.

VI. REFERENCES

1. Anderson E.C., Rowe L.W. The prevalence of antibody to the viruses of bovine virus diarrhoea, bovine herpes virus 1, rift valley fever, ephemeral fever and bluetongue and to *Leptospira sp* in free-ranging wildlife in Zimbabwe. Epidemiol. Infect. 1998. Oct;121(2):441-9.

2. Baker J.C. Bovine viral diarrhea virus: a review. J. Am. Vet. Med. Assoc. 1987. Jun 1;190(11):1449-58.

3. Baker J.C. The clinical manifestations of bovine viral diarrhea infection. Vet. Clin. North. Am. Food Anim. Pract. 1995. Nov;11(3):425-45.

4. Barkema H.W., Bartels C.J., van Wuijckhuise L., Hesselink J.W., Holzhauer M., Weber M.F., Franken P., Kock P.A, Bruschke C.J., Zimmer G.M. Outbreak of bovine virus diarrhea on Dutch dairy farms induced by a bovine herpesvirus 1 marker vaccine contaminated with bovine virus diarrhea virus type. Tijdschr Diergeneeskd. 2001. Mar 15;126(6):158-65.

5. Baule C., Kulcsár G., Belák K., Albert M., Mittelholzer C., Soós T., Kucsera L., Belák S. Pathogenesis of primary respiratory disease induced by isolates from a new genetic cluster of bovine viral diarrhea virus type I. J. Clin. Microbiol. 2001. Jan;39(1):146-53.

6. Baxi M., McRae D., Baxi S., Greiser-Wilke I., Vilcek S., Amoako K., Deregt D. A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhea viruses. Vet. Microbiol. 2006. Aug 25;116(1-3):37-44.

7. Beaudeau F., Belloc C., Seegers H., Assié S., Pourquier P., Joly A. Informative value of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of bovine viral diarrhoea virus (BVDV) antibodies in milk. J. Vet. Med. B. Infect. Dis. Vet. Public. Health. 2001. Nov;48(9):705-12.

8. Bezek D.M., Gröhn Y.T., Dubovi E.J. Effect of acute infection with noncytopathic or cytopathic bovine viral diarrhea virus isolates on bovine platelets. Am. J. Vet. Res. 1994. Aug;55(8):1115-9.

9. Bielanski A., Algire J., Lalonde A., Nadin-Davis S. Transmission of bovine viral diarrhea virus (BVDV) via in vitro-fertilized embryos to recipients, but not to their offspring. Theriogenology. 2008. Sep 30 (printing).

10. Birk A.V., Dubovi E.J., Cohen-Gould L., Donis R., Szeto H.H. Cytoplasmic vacuolization responses to cytopathic bovine viral diarrhoea virus. Virus Res. 2008. Mar;132(1-2):76-85.

11. Biuk-Rudan N., Cvetníc S., Madić J., Rudan D. Prevalence of antibodies to IBR and BVD viruses in dairy cows with reproductive disorders. Theriogenology. 1999. Apr. 1;51(5):875-81.

12. Bolin S.R., Matthews P.J., Ridpath J.F. Methods for detection and frequency of contamination of fetal calf serum with bovine viral diarrhea virus and antibodies against bovine viral diarrhea virus. J. Vet. Diagn. Invest. 1991. Jul;3(3):199-203.

13. Bolin S.R, Grooms D.L. Origination and consequences of bovine viral diarrhea virus diversity. Vet. Clin. North. Am. Food Anim. Pract. 2004. Mar;20(1):51-68.

14. Booker C.W., Abutarbush S.M., Morley P.S., Guichon P.T., Wildman B.K., Jim G.K., Schunicht O.C., Pittman T.J., Perrett T., Ellis J.A., Appleyard G., Haines D.M. The effect of bovine viral diarrhea virus infections on health and performance of feedlot cattle. Can. Vet. J. 2008. Mar;49(3):253-60.

15. Brackenbury L.S., Carr B.V., Charleston B. Aspects of the innate and adaptive immune responses to acute infections with BVDV. Vet. Microbiol. 2003. Nov 7;96(4):337-44.

16. Brewoo J.N., Haase C.J., Sharp P., Schultz R.D. Leukocyte profile of cattle persistently infected with bovine viral diarrhea virus. Vet. Immunol. Immunopathol. 2007. Feb 15;115(3-4):369-74.

17. Brock K.V., Grooms D.L., Ridpath J., Bolin S.R. Changes in levels of viremia in cattle persistently infected with bovine viral diarrhea virus. J Vet Diagn Invest. 1998. Jan;10(1):22-6.

18. Brock K.V. The persistence of bovine viral diarrhea virus. Biologicals. 2003. Jun;31(2):133-5.

19. Brock K.V. The many faces of bovine viral diarrhoea virus. Vet. Clin. North Am. Food Anim. Pract. 2004a. Mar;20(1):1-3.

20. Brock K.V. Strategies for the control and prevention of bovine viral diarrhea virus. Vet. Clin. North Am. Food Anim. Pract. 2004b. Mar;20(1):171-80.

21. Brock K.V., McCarty K., Chase C.C., Harland R. Protection against fetal infection with either bovine viral diarrhea virus type 1 or type 2 using a noncytopathic type 1 modified-live virus vaccine. Vet. Ther. 2006. Spring;7(1):27-34.

22. Brodersen B.W. Immunohistochemistry used as a screening method for persistent bovine viral diarrhea virus infection. Vet. Clin. North. Am. Food Anim. Pract. 2004. Mar;20(1):85-93.

23. Brownlie J., Hooper L.B., Thompson I., Collins M.E. Maternal recognition of fetal infection with bovine virus diarrhoea virus (BVDV)--the bovine pestivirus. Clin. Diagn. Virol. 1998. Jul 15;10(2-3):141-50.

24. Campbell J.R. Effect of bovine viral diarrhea virus in the feedlot. Vet. Clin. North. Am. Food Anim. Pract. 2004. Mar;20(1):39-50.

25. Cavirani S., Luini M., Allegri G., Fabbi G., Bottarelli E., Flamini C.F. Un decennio di ricerche sierologiche sulla diffusione di Bovine Herpesvirus 1 (BHV-1) Bovine Viral Diarrhea Virus (BVDV) e Bovine Herpesvirus 4 (BHV-4) in allevamenti bovini con turbe della fertilità. Selezione Veterinaria. 1992. 33, 459- 467.

26. Chase C.C., Elmowalid G., Yousif A.A. The immune response to bovine viral diarrhea virus: a constantly changing picture. Vet. Clin. North Am. Food Anim. Pract. 2004. Mar;20(1):95-114.

27. Chimeno Z.S., Leunda M.R., Odeón A., Taboga O. Recombinant E2 glycoprotein of bovine viral diarrhea virus induces a solid humoral neutralizing immune response but fails to confer total protection in cattle. Braz. J. Med. Biol. Res. 2007. Jun;40(6):813-8.

28. Ciulli S., Galletti E., Battilani M., Scagliarini A., Gentile A., Morganti L., Prosperi S. Genetic typing of bovine viral diarrhoea virus: evidence of an increasing number of variants in Italy. New. Microbiol. 2008. Apr;31(2):263-71.

29. Collen T., Morrison W.I. CD4(+) T-cell responses to bovine viral diarrhoea virus in cattle. Virus Res. 2000. Mar;67(1):67-80.

30. Collen T., Carr V., Parsons K., Charleston B., Morrison W.I. Analysis of the repertoire of cattle CD4(+) T cells reactive with bovine viral diarrhoea virus. Vet. Immunol. Immunopathol. 2002. Sep 10;87(3-4):235-8.

31. Cornish T.E., van Olphen A..L., Cavender J.L., Edwards J.M., Jaeger P.T., Vieyra L.L., Woodard L.F., Miller D.R., O'Toole D. Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detection of calves persistently infected with bovine viral diarrhea virus. J. Vet. Diagn. Invest. 2005. Mar;17(2):110-7.

32. Daly R., Hennings J., Graham T. BVD diagnostics at SDSU ADRDL: Demonstrating persistently infected cattle via antigen detection. Animal Health Matters. 2006. August 2: 3-6.

33. Dabak M., Karapinar T., Gulacti I., Bulut H., Kizil O., Aydin S. Hemorrhagic syndrome-like disease in calves with bovine viral diarrhea and mucosal disease complex. J. Vet. Intern. Med. 2007. May-Jun;21(3):514-8.

34. Dean H.J., Leyh R. Cross-protective efficacy of a bovine viral diarrhea virus (BVDV) type 1 vaccine against BVDV type 2 challenge. Vaccine. 1999. Mar 5;17(9-10):1117-24.

35. Deregt D., Carman P.S., Clark R.M., Burton K.M., Olson W.O., Gilbert S.A. A comparison of polymerase chain reaction with and without RNA extraction and virus isolation for detection of bovine viral diarrhea virus in young calves. J. Vet. Diagn. Invest. 2002. Sep;14(5):433-7.

36. Desport M., Collins M.E., Brownlie J. Genome instability in BVDV: an examination of the sequence and structural influences on RNA recombination. Virology. 1998. July 5;246(2):352-61.

37. Donis R.O., Corapi W.V., Dubovi E.J. Bovine viral diarrhea virus proteins and their antigenic analyses. Arch. Virol. Suppl. 1991. 3:29-40.

38. Drew T.W., Yapp F., Paton D.J. The detection of bovine viral diarrhoea virus in bulk milk samples by the use of a single-tube RT-PCR. Vet. Microbiol. 1999. Jan;64(2-3):145-54.

39. Dubovi E.J. Impact of bovine viral diarrhea virus on reproductive performance in cattle. Vet. Clin. North. Am. Food Anim. Pract. 1994. Nov;10(3):503-14.

40. Duncan C., Van Campen H., Soto S., LeVan I.K., Baeten L.A., Miller M.W. Persistent Bovine viral diarrhea virus infection in wild cervids of Colorado. J. Vet. Diagn. Invest. 2008. Sep;20(5):650-3.

41. Edmondson M.A., Givens M.D., Walz P.H., Gard J.A., Stringfellow D.A., Carson R.L. Comparison of tests for detection of bovine viral diarrhea virus in diagnostic samples. J. Vet. Diagn. Invest. 2007. Jul;19(4):376-81.

42. Ellis J.A., Martin K., Norman G.R., Haines D.M. Comparison of detection methods for bovine viral diarrhea virus in bovine abortions and neonatal death. J. Vet. Diagn. Invest. 1995. Oct;7(4):433-6.

43. Ellsworth M.A., Brown M.J., Fergen B.J., Ficken M.D., Tucker C.M., Bierman P., TerHune T.N. Safety of a modified-live combination vaccine against respiratory and reproductive diseases in pregnant cows. Vet. Ther. 2003. Summer;4(2):120-7.

44. Ellsworth M.A., Fairbanks K.K., Behan S., Jackson J.A., Goodyear M., Oien N.L., Meinert T.R., Leyh R.D. Fetal protection following exposure to calves persistently infected with bovine viral diarrhea virus type 2 sixteen months after primary vaccination of the dams. Vet. Ther. 2006. Fall;7(3):295-304.

45. Endsley J.J., Roth J.A, Ridpath J., Neill J. Maternal antibody blocks humoral but not T cell responses to BVDV. Biologicals. 2003. Jun;31(2):123-5.

46. Ezanno P., Fourichon C., Seegers H. Influence of herd structure and type of virus introduction on the spread of bovine viral diarrhoea virus (BVDV) within a dairy herd. Vet. Res. 2008. Sep-Oct;39(5):39.

47. Falcone E., Tollis M., Conti G. Bovine viral diarrhea disease associated with a contaminated vaccine. Vaccine. 1999. Oct 14;18(5-6):387-8.

48. Falcone E., Cordioli P., Sala G., Tarantino M., Tollis M. Genotyping of bovine viral diarrhoea viruses isolated from cattle in northern Italy. Vet. Res. Commun. 2001. Feb;25(2):161-7.

49. Falcone E., Cordioli P., Tarantino M., Muscillo M., La Rosa G., Tollis M. Genetic heterogeneity of bovine viral diarrhoea virus in Italy. Vet. Res. Commun. 2003. Sep;27(6):485-94.

50. Ferrari G., Scicluna M.T., Bonvicini D., Gobbi C., Della Verità F., Valentini A., Autorino G.L. Bovine virus diarrhoea (BVD) control programme in an area in the Rome province (Italy). Vet. Microbiol. 1999. Jan;64(2-3):237-45.

51. Fray M.D., Clarke M.C., Thomas L.H., McCauley J.W., Charleston B. Prolonged nasal shedding and viraemia of cytopathogenic bovine virus diarrhoea virus in experimental late-onset mucosal disease. Vet. Rec. 1998. Nov 28;143(22):608-11.

52. Fray M.D., Supple E.A., Morrison W.I., Charleston B. Germinal centre localization of bovine viral diarrhoea virus in persistently infected animals. J. Gen. Virol. 2000. Jul;81(Pt 7):1669-73.

53. Frey H.R., Eicken K., Grummer B., Kenklies S., Oguzoglu T.C., Moennig V. Foetal protection against bovine virus diarrhoea virus after two-step vaccination. J. Vet. Med. B. Infect. Dis. Vet. Public. Health. 2002. Dec;49(10):489-93.

54. Frölich K., Thiede S., Kozikowski T., Jakob W. A review of mutual transmission of important infectious diseases between livestock and wildlife in Europe. Ann. N. Y. Acad. Sci. 2002. Oct;969:4-13.

55. Fulton R.W., Saliki J.T., Burge L.J., Payton M.E. Humoral immune response and assessment of vaccine virus shedding in calves receiving modified live virus vaccines containing bovine herpesvirus-1 and bovine viral diarrhoea virus 1a. J. Vet. Med. B. Infect. Dis. Vet. Public. Health. 2003. Feb;50(1):31-7.

56. Gard J.A., Givens M.D., Stringfellow D.A. Bovine viral diarrhea virus (BVDV): epidemiologic concerns relative to semen and embryos. Theriogenology. 2007. Aug;68(3):434-42.

57. Garoussi M.T., Haghparast A., Estajee H. Prevalence of bovine viral diarrhoea virus antibodies in bulk tank milk of industrial dairy cattle herds in suburb of Mashhad-Iran. Prev. Vet. Med. 2008. Apr 17;84(1-2):171-6

58. Giammarioli M., Pellegrini C., Casciari C., Rossi E., De Mia G.M. Genetic diversity of bovine viral diarrhea virus 1: Italian isolates clustered in at least seven subgenotypes. J. Vet. Diagn. Invest. 2008. Nov;20(6):783-8.

59. Giangaspero M, Harasawa R, Weber L, Belloli A. Genoepidemiological evaluation of Bovine viral diarrhea virus 2 species based on secondary structures in the 5' untranslated region. J Vet Med Sci. 2008 Jun;70(6):571-80.

60. Gilbert SA, Burton KM, Prins SE, Deregt D. Typing of bovine viral diarrhea viruses directly from blood of persistently infected cattle by multiplex PCR. J Clin Microbiol. 1999 Jun;37(6):2020-3.

61. Givens MD, Heath AM, Carson RL, Brock KV, Edens MS, Wenzel JG, Stringfellow DA. Analytical sensitivity of assays used for detection of bovine viral diarrhea virus in semen samples from the Southeastern United States. Vet Microbiol. 2003 Oct 17;96(2):145-55.

62. Glew EJ, Carr BV, Brackenbury LS, Hope JC, Charleston B, Howard CJ. Differential effects of bovine viral diarrhoea virus on monocytes and dendritic cells. J Gen Virol. 2003 Jul;84(Pt 7):1771-80.

63. Gogorza LM, Moran PE, Larghi JL, Segui R, Lissarrague C, Saracco M, Braun M, Esteban EN. Detection of bovine viral diarrhea virus (BVDV) in seropositive cattle. Prev Vet Med. 2005 Nov 15;72(1-2):49-54.

64. Greiser-Wilke I., Grummer B., Moennig V. Bovine viral diarrhoea eradication and control programmes in Europe. Biologicals. 2003 Jun;31(2):113-8.

65. Gripshover E.M., Givens M.D., Ridpath J.F., Brock K.V., Whitley E.M., Sartin E.A. Variation in E(rns) viral glycoprotein associated with failure of immunohistochemistry and commercial antigen capture ELISA to detect a field strain of bovine viral diarrhea virus. Vet. Microbiol. 2007. Nov 15;125(1-2):11-21.

66. Grom J., Barlic-Maganja D. Bovine viral diarrhoea (BVD) infections--control and eradication programme in breeding herds in Slovenia. Vet. Microbiol. 1999. Jan;64(2-3):259-64.

67. Grooms D.L., Kaiser L., Walz P.H., Baker J.C. Study of cattle persistently infected with bovine viral diarrhea virus that lack detectable virus in serum. J. Am. Vet. Med. Assoc. 2001. Sep 1;219(5):629-31.

68. Grooms D.L., Keilen E.D. Screening of neonatal calves for persistent infection with bovine viral diarrhea virus by immunohistochemistry on skin biopsy samples. Clin. Diagn. Lab. Immunol. 2002. Jul;9(4):898-900.

69. Grooms D.L. Reproductive consequences of infection with bovine viral diarrhea virus. Vet Clin. North Am. Food Anim. Pract. 2004. Mar;20(1):5-19.

70. Grooms D.L., Bolin S.R., Coe P.H., Borges R.J., Coutu C.E. Foetal protection against continual exposure to bovine viral diarrhea virus following administration of a vaccine containing an inactivated bovine viral diarrhea virus fraction to cattle. Am. J. Vet. Res. 2007. Dec;68(12):1417-22.

71. Gunn G.J., Stott A.W., Humphry R.W. Modelling and costing BVD outbreaks in beef herds. Vet. J. 2004. Mar;167(2):143-9.

72. Hamers C., Dehan P., Couvreur B., Letellier C., Kerkhofs P., Pastoret P.P. Diversity among bovine pestiviruses. Vet. J. 2001. Mar;161(2):112-22.

73. Hilbe M., Arquint A., Schaller P., Zlinszky K., Braun U., Peterhans E., Ehrensperger F. Immunohistochemical diagnosis of persistent infection with Bovine Viral Diarrhea Virus (BVDV) on skin biopsies. Schweiz. Arch. Tierheilkd. 2007a. Aug;149(8):337-44.

74. Hilbe M., Stalder H., Peterhans E., Haessig M., Nussbaumer M., Egli C., Schelp C., Zlinszky K., Ehrensperger F. Comparison of five diagnostic methods for detecting bovine viral diarrhea virus infection in calves. J. Vet. Diagn. Invest. 2007b. Jan;19(1):28-34.

75. Hill FI., Reichel M.P., McCoy R.J., Tisdall D.J. Evaluation of two commercial enzyme-linked immunosorbent assays for detection of bovine viral diarrhoea virus in serum and skin biopsies of cattle. N. Z. Vet. J. 2007. Feb;55(1):45-8.

76. Houe H., Meyling A. Surveillance of cattle herds for bovine virus diarrhoea virus (BVDV)-infection using data on reproduction and calf mortality. Arch. Virol. Suppl. 1991.3:157-64.

77. Houe H. Epidemiology of bovine viral diarrhea virus. Vet. Clin. North Am. Food Anim. Pract. 1995. Nov;11(3):521-47.

78. Houe H. Economic impact of BVDV infection in dairies. Biologicals. 2003. Jun;31(2):137-43.

79. Houe H., Lindberg A., Moennig V. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. J. Vet. Diagn. Invest. 2006. Sep;18(5):427-36.

80. Hornberg A., Fernández S.R., Vogl C., Vilcek S., Matt M., Fink M., Köfer J., Schöpf K. Genetic diversity of *pestivirus* isolates in cattle from Western Austria. Vet. Microbiol. 2008. Sep 26. (printing)

81. Hult L., Lindberg A. Experiences from BVDV control in Sweden. Prev. Vet. Med. 2005. Nov 15;72(1-2):143-8

82. Kalaycioglu A.T. Bovine viral diarrhoea virus (BVDV) diversity and vaccination. A review. Vet. Q. 2007. Jun;29(2):60-7.

83. Kampa J., Ståhl K., Renström L.H., Alenius S. Evaluation of a commercial Erns-capture ELISA for detection of BVDV in routine diagnostic cattle serum samples. Acta Vet. Scand. 2007. Mar 13;49:7.

84. Kelling C.L. Evolution of bovine viral diarrhea virus vaccines. Vet. Clin. North Am. Food Anim. Pract. 2004. Mar;20(1):115-29.

85. Kelling C.L., Hunsaker B.D., Steffen D.J., Topliff C.L., Eskridge K.M. Characterization of protection against systemic infection and disease from experimental bovine viral diarrhea virus type 2 infection by use of a modified-live noncytopathic type 1 vaccine in calves. Am. J. Vet. Res. 2007. Jul;68(7):788-96.

86. Kennedy JA. Diagnostic efficacy of a reverse transcriptase-polymerase chain reaction assay to screen cattle for persistent bovine viral diarrhea virus infection. J. Am. Vet. Med. Assoc. 2006a. Nov 1;229(9):1472-4.

87. Kennedy J.A., Mortimer R.G., Powers B. Reverse transcription-polymerase chain reaction on pooled samples to detect bovine viral diarrhea virus by using fresh ear-notch-sample supernatants. J. Vet. Diagn. Invest. 2006b. Jan;18(1):89-93.

88. Kovács F., Magyar T., Rinehart C., Elbers K., Schlesinger K., Ohnesorge W.C. The live attenuated bovine viral diarrhea virus components of a multi-valent vaccine confer protection against fetal infection. Vet. Microbiol. 2003. Oct 17;96(2):117-31.

89. Kuhne S., Schroeder C., Holmquist G., Wolf G., Horner S., Brem G., Ballagi A. Detection of bovine viral diarrhoea virus infected cattle testing tissue samples derived from ear tagging using an E^{rns} capture ELISA.. J. Vet. Med. B. Infect. Dis. Vet. Public. Health. 2005. Aug;52(6):272-7.

90. Kuijk H., Franken P., Mars M.H., Weg W., Makoschey B. Monitoring of BVDV in a vaccinated herd by testing milk for antibodies to NS3 protein. Vet. Rec. 2008. Oct 18;163(16):482-4.

91. Kümmerer B.M., Tautz N., Becher P., Thiel H., Meyers G. The genetic basis for cytopathogenicity of pestiviruses. Vet. Microbiol. 2000. Nov 15;77(1-2):117-28.

92. Larson R.L., Groteloescher D.M., Brock K.V., Hunsaker B.D., Smith R.A., MacGregor D.S., Daragatz D.A. Bovine Viral Diarrea (BVD): Review for beef cattle veterinarians. Bov. Pract. 2004. 38: 93-102.

93. Larson R.L., Brodersen B.W., Groteloescher D.M., Bordett W., Brock K.V., Fulton R.W., Kennedy J.A., Daragatz D.A.. Considerations for Bovine Viral Diarrhea (BVD) testing. Bov. Pract. 2005. 39 (2): 96-100.

94. Larson R.L. Memories of Proceedings Planned BVD Control in Beef Herds. Production Level-Applied Science Session. Denver, Colorado. 2006. January 31.

95. Lee S.R., Nanduri B., Pharr G.T., Stokes J.V., Pinchuk L.M. Bovine Viral Diarrhea Virus infection affects the expression of proteins related to professional antigen presentation in bovine monocytes. Biochim. Biophys. Acta. 2009. Jan;1794(1):14-22.

96. Lee YM., Tscherne D.M., Yun S.I., Frolov I., Rice C.M. Dual mechanisms of *pestiviral* superinfection exclusion at entry and RNA replication. J. Virol. 2005. Mar;79(6):3231-42.

97. Letellier C., Kerkhofs P., Wellemans G., Vanopdenbosch E. Detection and genotyping of bovine diarrhea virus by reverse transcription-polymerase chain amplification of the 5' untranslated region. Vet. Microbiol. 1999. Jan;64(2-3):155-67.

98. Letellier C., Kerkhofs P. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. J. Virol. Methods. 2003. Dec;114(1):21-7.

99. Liang R., van den Hurk J.V., Landi A., Lawman Z., Deregt D., Townsend H., Babiuk L.A. DNA prime protein boost strategies protect cattle from bovine viral diarrhea virus type 2 challenge. J. Gen. Virol. 2008. Feb;89(Pt 2):453-66.

100. Liebler-Tenorio E.M., Ridpath J.F., Neill J.D. Lesions and tissue distribution of viral antigen in severe acute versus subclinical acute infection with BVDV2. Biologicals. 2003. Jun;31(2):119-22.

101. Liebler-Tenorio E.M., Ridpath J.E., Neill J.D. Distribution of viral antigen and tissue lesions in persistent and acute infection with the homologous strain of noncytopathic bovine viral diarrhea virus. J. Vet. Diagn. Invest. 2004. Sep;16(5):388-96.

102. Liebler-Tenorio E.M., Kenklies S., Greiser-Wilke I., Makoschey B., Pohlenz J.F. Incidence of BVDV1 and BVDV2 infections in cattle submitted for necropsy in Northern Germany. J. Vet. Med. B. Infect. Dis. Vet. Public Health. 2006. Oct;53(8):363-9.

103. Lindberg A.L., Alenius S. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet. Microbiol. 1999. Jan;64(2-3):197-222.

104. Lindberg A., Houe H. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control.Prev. Vet. Med. 2005. Nov 15;72(1-2):55-73.

105. Lindberg A., Brownlie J., Gunn G.J., Houe H., Moennig V., Saatkamp H.W., Sandvik T., Valle P.S. The control of bovine viral diarrhoea virus in Europe: today and in the future. Rev. Sci. Tech. 2006. Dec;25(3):961-79.

106. Loneragan G.H., Thomson D.U., Montgomery D.L., Mason G.L., Larson R.L. Prevalence, outcome, and health consequences associated with persistent infection with bovine viral diarrhea virus in feedlot cattle. J. Am. Vet. Med. Assoc. 2005. Feb 15;226(4):595-601.

107. Lunardi M., Headley S.A., Lisbôa J.A., Amude A.M., Alfieri A.A. Outbreak of acute bovine viral diarrhea in Brazilian beef cattle: clinicopathological findings and molecular characterization of a wild-type BVDV strain subtype 1b. Res. Vet. Sci. 2008. Dec;85(3):599-604.

108. Luzzago C., Piccinini R., Zepponi A., Zecconi A. Study on prevalence of bovine viral diarrhoea virus (BVDV) antibodies in 29 Italian dairy herds with reproductive problems. Vet. Microbiol. 1999. Jan;64(2-3):247-52.

109. Luzzago C., Bandi C., Bronzo V., Ruffo G., Zecconi A. Distribution pattern of bovine viral diarrhoea virus strains in intensive cattle herds in Italy. Vet. Microbiol. 2001. Nov 26;83(3):265-74.

110. Luzzago C., Frigerio M., Tolari F., Mazzei M., Salvadori C., Del Piero F., Arispici M. Indirect immunohistochemistry on skin biopsy for the detection of persistently infected cattle with bovine viral diarrhoea virus in Italian dairy herds. New Microbiol. 2006. Apr;29(2):127-31.

111. Luzzago C., Frigerio M., Piccinini R., Daprà V., Zecconi A. A scoring system for risk assessment of the introduction and spread of bovine viral diarrhoea virus in dairy herds in Northern Italy. Vet. J. 2008. Aug;177(2):236-41.

112. Mainar-Jaime R.C., Berzal-Herranz B., Arias P., Rojo-Vázquez F.A. Epidemiological pattern and risk factors associated with bovine viral-diarrhoea virus (BVDV) infection in a non-vaccinated dairy-cattle population from the Asturias region of Spain. Prev. Vet. Med. 2001. Nov 2;52(1):63-73.

113. Makoschey B., Janssen M.G., Vrijenhoek M.P., Korsten J.H., Marel P. An inactivated bovine virus diarrhoea virus (BVDV) type 1 vaccine affords clinical protection against BVDV type 2. Vaccine. 2001. Apr 30;19(23-24):3261-8.

114. Mars M.H., Bruschke C.J., van Oirschot J.T. Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions. Vet. Microbiol. 1999. Apr 19;66(3):197-207.

115. Mars M.H., Van Maanen C. Diagnostic assays applied in BVDV control in The Netherlands. Prev. Vet. Med. 2005. Nov 15;72(1-2):43-8.

116. Moen A., Sol J., Sampimon O. Indication of transmission of BVDV in the absence of persistently infected (PI) animals. Prev. Vet. Med. 2005. Nov 15;72(1-2):93-8.

117. Moennig V., Houe H., Lindberg A. BVD control in Europe: current status and perspectives. Anim. Health. Res. Rev. 2005a. Jun;6(1):63-74.

118. Moennig V., Eicken K., Flebbe U., Frey H.R., Grummer B., Haas L., Greiser-Wilke I., Liess B. Implementation of two-step vaccination in the control of bovine viral diarrhoea (BVD). Prev. Vet. Med. 2005b. Nov 15;72(1-2):109-14.

119. Moennig V., Houe H., Lindberg A. BVD Control: State of the art. Memories of World Buatrics Congress. Nice, France. 2006. p: 94-102.

120. Moerman A., Straver P.J., de Jong M.C., Quak J., Baanvinger T., van Oirschot J.T. A long term epidemiological study of bovine viral diarrhoea infections in a large herd of dairy cattle. Vet. Rec. 1993. Jun 19;132(25):622-6.

121. Moerman A., Straver P.J., de Jong M.C., Quak J., Baanvinger T., van Oirschot J.T. Clinical consequences of a bovine virus diarrhoea virus infection in a dairy herd: a longitudinal study. Vet. Q. 1994. Jul;16(2):115-9.

122. Mockeliū niene V., Salomskas A., Mockeliū nas R., Petkevicius S. Prevalence and epidemiological features of bovine viral diarrhoea virus infection in Lithuania. Vet. Microbiol. 2004. Mar 26;99(1):51-7.

123. Muñoz-Zanzi C.A., Johnson W.O., Thurmond M.C., Hietala S.K. Pooled-sample testing as a herd-screening tool for detection of bovine viral diarrhea virus persistently infected cattle. J. Vet. Diagn. Invest. 2000. May;12(3):195-203.

124. Muñoz-Zanzi C.A., Hietala S.K., Thurmond M.C., Johnson W.O. Quantification, risk factors, and health impact of natural congenital infection with bovine viral diarrhea virus in dairy calves. Am. J. Vet. Res. 2003. Mar;64(3):358-65.

125. Muñoz-Zanzi C.A., Thurmond M., Hietala S., Johnson W. Factors affecting sensitivity and specificity of pooled-sample testing for diagnosis of low prevalence infections. Prev. Vet. Med. 2006. Jun 16;74(4):309-22.

126. Murray C.L., Marcotrigiano J., Rice C.M. Bovine viral diarrhoea virus core is an intrinsically disordered protein that binds RNA. J. Virol. 2008. Feb;82(3):1294-304.

127. Neill J.D., Ridpath J.F., Lange A., Zuerner R.L. Bovine viral diarrhoea virus infection alters global transcription profiles in bovine endothelial cells. Dev. Biol (Basel). 2008. 132:93-8.

128. Nielsen S.S., Roensholt L., Bitsch V. Bovine virus diarrhea virus in free-living deer from Denmark. J. Wildl. Dis. 2000. Jul;36(3):584-7.

129. Niskanen R., Alenius S., Larsson B., Jacobsson S.O. Determination of level of antibodies to bovine virus diarrhoea virus (BVDV) in bulk tank milk as a tool in the diagnosis and prophylaxis of BVDV infections in dairy herds. Arch. Virol. Suppl. 1991.3:245-51.

130. Niskanen R. Relationship between the levels of antibodies to bovine viral diarrhoea virus in bulk tank milk and the prevalence of cows exposed to the virus. Vet. Rec. 1993. Oct 2;133(14):341-4.

131. Niskanen R., Lindberg A. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. Vet. J. 2003. Mar;165(2):125-30.

132. Niza-Ribeiro J., Pereira A., Souza J., Madeira H., Barbosa A., Afonso C. Estimated BVDV-prevalence, -contact and -vaccine use in dairy herds in Northern Portugal. Prev. Vet. Med. 2005. Nov 15;72(1-2):81-5

133. Njaa B.L., Clark E.G., Janzen E., Ellis J.A., Haines D.M. Diagnosis of persistent bovine viral diarrhea virus infection by immunohistochemical staining of formalin-fixed skin biopsy specimens. J. Vet. Diagn. Invest. 2000. Sep;12(5):393-9.

134. Nuotio L., Juvonen M., Neuvonen E., Sihvonen L., Husu-Kallio J. Prevalence and geographic distribution of bovine viral diarrhoea (BVD) infection in Finland 1993-1997. Vet. Microbiol. 1999. Jan;64(2-3):231-5.

135. Obando C., Baule C., Pedrique C., Veracierta C., Belák S., Merza M., Moreno-Lopez J. Serological and molecular diagnosis of bovine viral diarrhoea virus and evidence of other viral infections in dairy calves with respiratory disease in Venezuela. Acta. Vet. Scand. 1999. 40(3):253-62.

136. Odeon A.C., Risatti G., Kaiser G.G., Leunda M.R., Odriozola E., Campero C.M., Donis R.O. Bovine viral diarrhea virus genomic associations in mucosal disease, enteritis and generalized dermatitis outbreaks in Argentina. Vet. Microbiol. 2003. Oct 17;96(2):133-44.

137. Oguzoglu T.C., Frey H.R., Eicken K., Grummer B., Liess B., Moennig V. Kinetics and persistence of neutralizing antibodies against bovine viral diarrhea virus-1 and -2 and border disease virus after two step vaccination of cattle. Dtsch. Tierarztl. Wochenschr. 2003. Jan;110(1):14-7.

138. Olafson P. Hyperkeratosis (X disease) of cattle. Cornell Vet. 1947. Oct;37(4):279-91.

139. Palfi V., Houe H., Philipsen J. Studies on the decline of bovine virus diarrhoea virus (BVDV) maternal antibodies and detectability of BVDV in persistently infected calves. Acta. Vet. Scand. 1993.34(1):105-7.

140. Pankraz A., Thiel H.J., Becher P. Essential and nonessential elements in the 3' non-translated region of Bovine viral diarrhoea virus. J. Virol. 2005. Jul;79(14):9119-27.

141. Pellerin C., van den Hurk J., Lecomte J., Tussen P. Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology. 1994. Sep;203(2):260-8.

142. Perry G.H. Risk assessment of transmission of bovine viral diarrhea virus (BVDV) in abattoir-derived in vitro produced embryos. Theriogenology. 2007. Jul 1;68(1):38-55.

143. Peterhans E., Jungi T.W., Schweizer M. BVDV and innate immunity. Biologicals. 2003. Jun;31(2):107-12.

144. Piccinini R., Luzzago C., Frigerio M., Daprà V., Liandris E., Zecconi A. Comparison of blood non-specific immune parameters in Bovine virus diarrhoea virus (BVDV) persistently infected and in immune heifers. J. Vet. Med. B. Infect. Dis. Vet. Public. Health. 2006. Mar;53(2):62-7.

145. Platt R., Coutu C., Meinert T., Roth JA. Humoral and T cell-mediated immune responses to bivalent killed bovine viral diarrhea virus vaccine in beef cattle. Vet. Immunol. Immunopathol. 2008. Mar 15;122(1-2):8-15.

146. Plavsic M.Z., Prodafikas G. Evaluation of a new sandwich enzyme-linked immunosorbent assay for detection of bovine viral diarrhea virus in unprocessed fetal bovine serum. J. Vet. Diagn. Invest. 2001. May;13(3):261-2.

147. Potgieter L.N. Immunology of bovine viral diarrhea virus. Vet. Clin. North Am. Food. Anim. Pract. 1995. Nov;11(3):501-20.

148. Reber A.J., Tanner M., Okinaga T., Woolums A.R., Williams S., Ensley D.T., Hurley D.J. Evaluation of multiple immune parameters after vaccination with modified live or killed bovine viral diarrhea virus vaccines. Comp. Immunol. Microbiol. Infect. Dis. 2006. Jan;29(1):61-77.

149. Reichel M.P., Hill F.I., Voges H. Does control of bovine viral diarrhoea infection make economic sense? N. Z. Vet. J. 2008. Apr;56(2):60-6.

150. Ridpath J.F., Bolin S.R, Dubovi E.J. Segregation of bovine viral diarrhea virus into genotypes. Virology. 1994. Nov 15;205(1):66-74.

151. Ridpath J.F., Neill J.D. Detection and characterization of genetic recombination in cytopathic type 2 bovine viral diarrhea viruses. J. Virol. 2000. Sep;74(18):8771-4.

152. Ridpath J.F., Hietala S.K., Sorden S., Neill J.D. Evaluation of the reverse transcription-polymerase chain reaction/probe test of serum samples and immunohistochemistry of skin sections for detection of acute bovine viral diarrhea infections. J. Vet. Diagn. Invest. 2002. Jul;14(4):303-7.

153. Ridpath J.F. BVDV genotypes and biotypes: practical implications for diagnosis and control. Biologicals. 2003. Jun;31(2):127-31.

154. Ridpath J.F., Bendfeldt S., Neill J.D., Liebler-Tenorio E. Lymphocytopathogenic activity in vitro correlates with high virulence in vivo for BVDV type 2 strains: Criteria for a third biotype of BVDV. Virus Res. 2006a. Jun;118(1-2):62-9.

155. Ridpath J.F., Neill J.D., Vilcek S., Dubovi E.J., Carman S. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Vet. Microbiol. 2006b. May 31;114(3-4):196-204.

156. Rikula U., Nuotio L., Aaltonen T., Ruoho O. Bovine viral diarrhoea virus control in Finland 1998-2004. Prev. Vet. Med. 2005. Nov 15;72(1-2):139-42.

157. Rikula U., Nuotio L., Laamanen U.I., Sihvonen L. Transmission of bovine viral diarrhoea virus through the semen of acutely infected bulls under field conditions. Vet. Rec. 2008. Jan 19;162(3):79-82.

158. Riond J.L., Cullen J.M., Godfrey V.L., Hunt E.L., Anderson K.L. Bovine viral diarrhea virus-induced cerebellar disease in a calf. J. Am. Vet. Med. Assoc. 1990. Dec 15;197(12):1631-2.

159. Rossmanith W., Janacek R., Wilhelm E. Control of BVDV-infection on common grassland--the key for successful BVDV-eradication in Lower Austria. Prev. Vet. Med. 2005. Nov 15;72(1-2):133-7.

160. Saliki J.T., Huchzermeier R., Dubovi E.J. Evaluation of a new sandwich ELISA kit that uses serum for detection of cattle persistently infected with BVD virus. Ann. N. Y. Acad. Sci. 2000. 916:358-63.

161. Saliki J.T, Dubovi E.J. Laboratory diagnosis of bovine viral diarrhea virus infections. Vet. Clin. North Am. Food Anim. Pract. 2004. Mar;20(1):69-83.

162. Sandvik T., Krogsrud J. Evaluation of an antigen-capture ELISA for detection of bovine viral diarrhea virus in cattle blood samples. J. Vet. Diagn. Invest. 1995. Jan;7(1):65-71.

163. Sandvik T. Progress of control and prevention programs for bovine viral diarrhea virus in Europe. Vet. Clin. North Am. Food Anim. Pract. 2004. Mar;20(1):151-69.

164. Sandvik T. Selection and use of laboratory diagnostic assays in BVD control programmes. Prev. Vet. Med. 2005. Nov 15;72(1-2):3-16.

165. Seki Y., Seimiya Y.M., Yaegashi G., Sato C. Identification of herds with cattle persistently infected with bovine viral diarrhea virus by virological evaluation of three calves. J. Vet. Med. Sci. 2006. Mar;68(3):255-8.

166. Seki Y., Seimiya Y.M., Motokawa M., Miyazaki H., Konno M., Yaegashi G. Field application of the combination of neutralizing test and virus isolation, so-called spot test, to detect herds including cattle persistently infected with bovine viral diarrhea virus. J. Vet. Med. Sci. 2007. Oct;69(10):1087-9.

167. Schefers J., Munoz-Zanzi C., Collins J.E., Goyal S.M., Ames T.R. Serological evaluation of precolostral serum samples to detect Bovine viral diarrhea virus infections in large commercial dairy herds. J. .Vet. Diagn. Invest. 2008. Sep;20(5):625-8.

168. Schweizer M., Mätzener P., Pfaffen G., Stalder H., Peterhans E. "Self" and "nonself" manipulation of interferon defense during persistent infection: bovine viral diarrhea virus resists alpha/beta interferon without blocking antiviral activity against unrelated viruses replicating in its host cells. J. Virol. 2006. Jul;80(14):6926-35.

169. Shin T., Acland H. Tissue distribution of bovine viral diarrhea virus antigens in persistently infected cattle. J. Vet .Sci. 2001. Aug;2(2):81-4.

170. Shoemaker M.L., Smirnova N.P., Bielefeldt-Ohmann H., Austin K.J., van Olphen A., Clapper J.A., Hansen T.R. Differential Expression of the Type I Interferon Pathway during Persistent and Transient Bovine Viral Diarrhea Virus Infection. J. Interferon Cytokine Res. 2008. Nov 17 (printing).

171. Smirnova N.P., Bielefeldt-Ohmann H., Van Campen H., Austin K.J., Han H., Montgomery D.L., Shoemaker M.L., van Olphen A.L., Hansen T.R. Acute non-cytopathic bovine viral diarrhea virus infection induces pronounced type I interferon response in pregnant cows and foetuses. Virus Res. 2008. Mar;132(1-2):49-58.

172. Smith D.R., Grotelueschen D.M. Biosecurity and biocontainment of bovine viral diarrhea virus. Vet. Clin. North Am. Food Anim. Pract. 2004. Mar;20(1):131-49.

173. Smith R.L., Sanderson M.W., Walz P.H., Givens M.D. Sensitivity of polymerase chain reaction for detection of bovine viral diarrhea virus in pooled serum samples and use of pooled polymerase chain reaction to determine prevalence of bovine viral diarrhea virus in auction market cattle. J. Vet. Diagn. Invest. 2008. Jan;20(1):75-8.

174. Sol J., Schaake J., Leloup M.R. Severe course of a primary BVD infection in a BVD-seronegative dairy herd. Tijdschr. Diergeneeskd. 1989. Sep 1;114(17):886-90.

175. Solis-Calderon J.J., Segura-Correa V.M., Segura-Correa J.C. Bovine viral diarrhoea virus in beef cattle herds of Yucatan, Mexico: seroprevalence and risk factors. Prev. Vet. Med. 2005. Dec 12;72(3-4):253-62.

176. Ståhl K., Rivera H., Vågsholm I., Moreno-López J. Bulk milk testing for antibody seroprevalences to BVDV and BHV-1 in a rural region of Peru. Prev. Vet. Med. 2002. Dec 30;56(3):193-202.

177. Ståhl K., Kampa J., Baule C., Isaksson M., Moreno-López J., Belák S., Alenius S., Lindberg A. Molecular epidemiology of bovine viral diarrhoea during the final phase of the Swedish BVD-eradication programme. Prev. Vet. Med. 2005. Nov 15;72(1-2):103-8.

178. Ståhl K., Kampa J., Alenius S., Persson W.A., Baule C., Aiumlamai S., Belák S. Natural infection of cattle with an atypical 'HoBi'-like pestivirus—implications for BVD control and for the safety of biological products. Vet. Res. 2007. May-Jun;38(3):517-23.

179. Ståhl K., Lindberg A., Rivera H., Ortiz C., Moreno-López J. Self-clearance from BVDV infections--a frequent finding in dairy herds in an endemically infected region in Peru. Prev. Vet. Med. 2008. Mar 17;83(3-4):285-96.

180. Stringfellow D.A., Riddell K.P., Givens M.D., Galik P.K., Sullivan E., Dykstra C.C., Robl J., Kasinathan P. Bovine viral diarrhea virus (BVDV) in cell lines used for somatic cell cloning. Theriogenology. 2005. Mar 1;63(4):1004-13.

181. Stoffregen B., Bolin S.R., Ridpath J.F., Pohlenz J. Morphologic lesions in type 2 BVDV infections experimentally induced by strain BVDV2-1373 recovered from a field case. Vet. Microbiol. 2000. Nov 15;77(1-2):157-62.

182. Sun J.H., Lemm J.A., O'Boyle D.R. Racela J., Colonno R., Gao M. Specific inhibition of bovine viral diarrhoea virus replicase. J. Virol. 2003. Jun;77(12):6753-60.

183. Swasdipan S., McGowan M., Phillips N., Bielefeldt-Ohmann H. Pathogenesis of transplacental virus infection: pestivirus replication in the placenta and fetus following respiratory infection. Microb. Pathog. 2002. Feb;32(2):49-60.

184. Tajima M., Ohsaki T., Okazawa M., Yasutomi I. Availability of oral swab sample for the detection of bovine viral diarrhea virus (BVDV) gene from the cattle persistently infected with BVDV. Jpn. J. Vet. Res. 2008. May;56(1):3-8

185. Talafha A.Q., Hirche S.M., Ababneh M.M., Al-Majali A.M., Ababneh M.M. Prevalence and risk factors associated with bovine viral diarrhea virus infection in dairy herds in Jordan. Trop. Anim. Health Prod. 2008. Jul 25.

186. Tautz N., Meyers G., Thiel HJ. Pathogenesis of mucosal disease, a deadly disease of cattle caused by a pestivirus. Clin. Diagn. Virol. 1998. Jul 15;10(2-3):121-7.

187. Tautz N., Thiel H.J. Cytopathogenicity of *pestiviruses*: cleavage of bovine viral diarrhoea virus NS2-3 has to occur at a defined position to allow viral replication. Arch. Virol. 2003. Jul;148(7):1405-12.

188. Thobokwe G., Heuer C., Hayes D.P. Validation of a bulk tank milk antibody ELISA to detect dairy herds likely infected with bovine viral diarrhoea virus in New Zealand. N. Z. Vet. J. 2004. Dec;52(6):394-400.

189. Thür B., Zlinszky K., Ehrensperger F. Immunohistochemical detection of bovine viral diarrhea virus in skin biopsies: a reliable and fast diagnostic tool. Zentralbl. Veterinarmed. B. 1996. May;43(3):163-6.

190. Toplak I., Sandvik T., Barlic-Maganja D., Grom J, Paton DJ. Genetic typing of bovine viral diarrhoea virus: most Slovenian isolates are of genotypes 1d and 1f. Vet. Microbiol. 2004. Apr 19;99(3-4):175-85.

191. Underdahl N.R., Grace O.D., Hoerlein A.B. Cultivation in tissue-culture of cytopathogenic agent from bovine mucosal disease. Proc. Soc. Exp. Biol. Med. 1957. Apr;94(4):795-7.

192. Valle P.S., Martin S.W., Tremblay R., Bateman K. Factors associated with being a bovine-virus diarrhoea (BVD) seropositive dairy herd in the Møre and Romsdal County of Norway. Prev. Vet. Med. 1999. Jun 11;40(3-4):165-77.

193. VanLeeuwen J.A., Keefe G.P., Tremblay R., Power C., Wichtel J.J. Seroprevalence of infection with Mycobacterium avium subspecies paratuberculosis, bovine leukemia virus, and bovine viral diarrhea virus in maritime Canada dairy cattle. Can. Vet. J. 2001. Mar;42(3):193-8.

194. Vilcek S., Björklund H.V., Horner G.W., Meers J., Belák S. Genetic typing of *pestiviruses* from New Zealand. N. Z. Vet. J. 1998. Feb;46(1):35-7.

195. Vilcek S., Paton D.J., Rowe L.W., Anderson E.C. Typing of *pestiviruses* from eland in Zimbabwe. J. Wildl. Dis. 2000. Jan;36(1):165-8.

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

197. Vilcek S., Durkovic B., Kolesarova M., Paton D.J. Genetic diversity of BVDV: consequences for classification and molecular epidemiology. Prev. Vet. Med. 2005. Nov 15;72(1-2):31-5.

198. Waldner C.L., Campbell J.R. Use of serologic evaluation for antibodies against bovine viral diarrhea virus for detection of persistently infected calves in beef herds. Am. J. Vet. Res. 2005. May;66(5):825-34.

199. Walz P.H., Bell T.G., Wells J.L., Grooms D.L., Kaiser L., Maes R.K., Baker J.C. Relationship between degree of viremia and disease manifestation in calves with experimentally induced bovine viral diarrhea virus infection. Am. J. Vet. Res. 2001. Jul;62(7):1095-103.

200. Weinstock D., Bhudevi B., Castro A.E. Single-tube single-enzyme reverse transcriptase PCR assay for detection of bovine viral diarrhea virus in pooled bovine serum. J. Clin. Microbiol. 2001. Jan;39(1):343-6.

201. Wilhelmsen C.L., Bolin S.R., Ridpath J.F., Cheville N.F., Kluge J.P. Lesions and localization of viral antigen in tissues of cattle with experimentally induced or naturally acquired mucosal disease, or with naturally acquired chronic bovine viral diarrhea. Am. J. Vet. Res. 1991. Feb;52(2):269-75.

202. Wittum T.E., Grotelueschen D.M., Brock K.V., Kvasnicka W.G., Floyd J.G., Kelling C.L., Odde K.G. Persistent bovine viral diarrhoea virus infection in US beef herds. Prev. Vet. Med. 2001. Apr 13;49(1-2):83-94.

203. Yamane D., Nagai M., Ogawa Y., Tohya Y., Akashi H. Enhancement of apoptosis via an extrinsic factor, TNF-alpha, in cells infected with cytopathic bovine viral diarrhea virus. Microbes Infect. 2005. Dec;7(15):1482-91.

204. Young N.J., Thomas C.J., Thompson I., Collins M.E., Brownlie J. Immune responses to non-structural protein 3 (NS3) of bovine viral diarrhoea virus (BVDV) in NS3 DNA vaccinated and naturally infected cattle. Prev. Vet. Med. 2005. Nov 15;72(1-2):115-20

205. Young N.J., Thomas C.J., Collins M.E., Brownlie J. Real-time RT-PCR detection of Bovine Viral Diarrhoea virus in whole blood using an external RNA reference. J. Virol. Methods. 2006. Dec;138(1-2):218-22.

206. Zhang S., Troyer D.L., Kapil S., Zheng L., Kennedy G., Weiss M., Xue W., Wood C., Minocha H.C. Detection of proviral DNA of bovine immunodeficiency virus in bovine tissues by polymerase chain reaction (PCR) and PCR in situ hybridization. Virology. 1997. Sep 29;236(2):249-57.

207. Zimmer G.M., Wentink G.H., Bruschke C., Westenbrink F.J., Brinkhof J., de Goey I. Failure of foetal protection after vaccination against an experimental infection with bovine virus diarrhea virus. Vet. Microbiol. 2002. Nov 6;89(4):255-65.

208. Zimmer G.M., Van Maanen C., De Goey I., Brinkhof J., Wentink G.H. The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. Vet. Microbiol. 2004. Jun 3;100(3-4):145-9.

VII. Annex No. 1 (Page 1)

EPIDEMIOLOGICAL FORM

1. GENERAL INFORMATION

Reception date_____ Reg. num_____
No. of samples received _____

2. Samples

Serum Plasma Blood Tissue Other: _____

3. Name of the farm / Address / Telephone:_____

4.Name of the Veterinarian / Address/ Telephone: _____

5. Herd Size / Zootecnic function: _____

6. Vaccination

IBR BVDV PI3 Others: _____

MLV Inactivated Polivalent Monovalent No vaccination

Last vaccination: _____

Vaccination program: _____

7. Clinical signs

Clinical history of the farm: _____

% Abortion_____
Reproductive disorders _____
Respiratory disorders _____
Others _____

8. Have you had BVDV in your farm? _____

9. Have some animals been introduced in the farm? When? _____

10. Have you tested new animals to detect BVDV?_____

11. There are farms near? (si / no / km) _____

10. Post-mortem inspection
Macroscopic/ histophatological founds: _____

11.**Other information**: _____

GENERAL INFROMATION OF ANIMALS (Page 2)

			Age (months)		Sex	Origin		Physiol. Status			
	ID	**Breed**	**<24**	**>24**	**M/F**	**B**	**P**	**P**	**NP**	**BVDV Ab**	**BVDV Ag**
1											
2											
3											
4											
5											
6											
7											
8											
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40											

Origin: B: born in the farm P: purchased
Physiologic status: P: pregnant NP: no pregnant
BVDV Ab: ELISA Antibody test
BVDV Ag: ACE

AURORA ROMERO TEJEDA

ACADEMIC ACTIVITIES

2006-2008

From January of 2006 to December of 2008

Activities:

- Development of a research project aimed at the evaluation of diagnostic methods to detect Bovine Viral Diarrhea affected cattle.
- Optimizing serological and PCR protocols to detect bacterial and viral pathogens responsible for infectious diseases of different animals.
- Collaboration in a research group to develop works and their presentation in international congresses.
- Collaboration as a member of the examination Commission for the " Microbiology" and " Animal Infectious diseases" courses.

Place: Infectious Diseases Laboratory. Faculty of Veterinary Medicine, University of Camerino.

Responsible: DVM, Dr. Silvia Preziuso.

From April 2th to May 30 of 2008

Activities:

- To observe and participate in activities concerning the different diagnostic procedures and their applications to Bovine Viral Diarrhoea (BVD) control with special emphasis on the development of the Colorado Voluntary BVD Control program

Place: Veterinary Diagnostic Laboratory. Rocky Ford, Colorado (United States of America).

Responsible: DVM, MS. James A. Kennedy.

Scientific Publication in International Congresses

1. Cuteri V., Attili A.R, Cittadini F., Preziuso S., **Romero Tejeda A**., Marenzoni M.L, Valente C. Selected agents involved in Buffalo (*Bubalus bubalis*) abortion in central Italy. XIV Congreso Internacional de la Federación Mediterránea de Sanidad y Producción de Rumiantes (FEMESPRUM). From 12 to 15 of July of 2006, Lugo-Santiago de Compostela, Spain.

2. Cuteri V., Cittadini F., **Romero Tejeda A**., Preziuso S., Marenzoni M.L, Passamonti F., Attili A.R, Giorgi G., Valente C. Interaction between BVDV and BoHV-1 infection in dairy herds in Italy. XIV Congreso Internacional de la Federación Mediterránea de Sanidad y Producción de Rumiantes (FEMESPRUM). From 12 to 15 of July of 2006, Lugo-Santiago de Compostela, Spain.

3. Cuteri V., **Romero Tejeda A**., Splendiani F., Valente C., Preziuso S. PCR identification of the virulence plasmids of *Rhodococcus equi* isolated from horses in Italy. LXI Convegno Nazionale della Società Italiana delle Scienze Veterinarie (SISVET). Salsomaggiore Terme (PR), Italy. September 26 to 29 of 2007.

4. **Romero Tejeda A.**, Thiry J., Preziuso S., Thiry E., Petralia P., Valente C., Cuteri V. Serological detection of a *Bovine Herspevirus 1* related infection in buffaloes (*Bubalus Bubalis*) by using BoHV-1 Blocking ELISA. XVI Congress of the Mediterranean Federation for Health and production of Ruminants (FeMeSPrum), April 22-26 of 2008. Zadar, Croatia.

5. **Romero Tejeda A.,** Thiry J., Preziuso S., Thiry E., Cuteri V. Bovine Herpesvirus 1 Related Infection in Buffaloes (*Bubalus Bubalis*): Serological Detection by Using Two Different BoHV-1 Blocking ELISA. XXV World Buiatrics Congress, July 6-11 2008. Budapest ,Hungary.

6. Cuteri V., Nisoli L., Fruganti A., **Romero Tejeda A**., Attili A. Evaluation of Diarrhoea Treatment with Injectable Enrofloxacin (Baytril® 5% Bayer) and Oral Rehydration Solution (Glutellac® Bayer) in Newborn Lactating Calves. XXV World Buiatrics Congress, July 6-11 of 2008. Budapest, Hungary.

7. Cuteri V., Froyman R., Nisoli L., **Romero Tejeda A**., Preziuso S., Attili A. Field Trial in Northern Italy to Evaluate the Efficacy of Two Different Therapies against Acute Clinical Mastitis in Dairy Cows. XXV World Buiatrics Congress, July 6-11 of 2008. Budapest, Hungary.

8. Cuteri V., Nisoli L., Attili A., **Romero Tejeda A**., Fruganti A. Clinical Field Evaluation of a Butaphosfan + Vitamin B12 Compound (Phosphorum B12/Catosal® – Bayer) in the Treatment of Subclinical Ketosis in Dairy Cows. XXV World Buiatrics Congress, July 6-11 of 2008. Budapest, Hungary.

9. **Romero Tejeda A**., Preziuso S., Valente C., Cuteri V. Study of BVDV seroprevalence and identification of persistent infected animals in three regions from central Italy. The 7th ESVV Pestivirus Symposium. Uppsala, Sweden, 6-19 September 2008. *Accepted with a grant

DEDICADO A

DIOS, por haberme bendecido con el regalo más preciado: la vida misma.

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