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Review Article

Diagnostic Approaches for Detection of Bovine Viral Diarrhea Virus Persistent Infection

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ABSTRACT

Bovine viral diarrhea is one of the most important diseases of cattle which is causing continuous economic losses to the cattle industry primarily due to decreased reproductive performance. Without doubt, direct contact between BVDV persistently infected, and susceptible animals is the most important transmission route of virus. All control programs which are in use in many countries of the world, mainly depend upon the detection of PI animals, eliminating them and preventing their return into the herds. Various diagnostic tests using ear notch biopsies and serum samples are in use with certain advantages and disadvantages. In detecting the BVDV persistent infection, a complete agreement (P value = 1) was observed between Real time RT–PCR, AC–ELISA, VI and IHC. All four assays were found specific but real time RT–PCR was found to be more sensitive. Both, VI and IHC were found labour intensive, as diagnosis may take more than one week to be made. Further peroxidase based IHC interpretation was found to be difficult somewhat due to subjective reasoning. It is generally recognized, that real time RT–PCR is more sensitive for BVDV detection than the other methods employed in the study period. However, use of real time RT–PCR for screening of individual animal cases is cost prohibited. AC–ELISA while relatively less sensitive was shown to has sufficient sensitivity for cost effective identification of PI. The results appear to warrant the use of AC–ELISA on ear notch biopsies for routine diagnosis of PI animals followed by real time RT–PCR on suspicious samples.

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INTRODUCTION

Bovine viral diarrhea (BVD) is considered as one of the most commonly encountered and economically important viral infection of cattle in many countries of the world (Childs, 1946; Corapi et al., 1990b; Evermann et al., 1993). Economic losses associated with BVDV infection appear to be mainly due to reproductive disorders (Brownlie et al., 1989; Woodard, 1994; Saliki, 1996). The estimated economic losses, ranging from few thousand up to $100000, in an individual cow dairy herd outbreaks with highly pathogenic strains, have been reported (Houe, 2003; Alves et al., 1996). A key element in maintaining the BVDV infection in cattle herds is the presence of PI animals (Niskanen et al., 2000; Traven et al., 1991). All control programs which are in use in many countries of the world, mainly depend upon the detection and removal of PI animals, and prevention of introduction of PI animals in the herds. Cost effectiveness of tests under consideration is an important criterion. A prevalence of PI animals ranging from 0.5 to 2% has been reported by many workers (Harkness et al., 1978; Meyling, 1984; Howard et al., 1986), therefore every member of a herd must be tested. Therefore, there is a need to identify adequate methods for the detection and identification of the persistent animals in a timely and accurate manner. Detection of PI animals, at young ages, is of major benefit to cattle farmers who wish to implement BVDV control programs.

Currently, various diagnostic techniques are being used, for identification of persistently infected animals. Inspite of that, BVD virus is still circulating on the farms. Continuous presence of BVD virus, on the cattle farms, is mainly due to three reasons; subclinical nature of the infections; hesitation of cattle producers to participate in the control program, as, testing of all animals on an average sized cattle farm, containing approximately 100 animals, is not cost effective, and lastly, some diagnostic techniques (virus isolation) may be invalidated by the presence of maternally derived colostral antibodies leading to false positive results.

To achieve successful prevention and control of an infectious disease, there must be adequate methods for the diagnostic detection and identification of the pathogen in a timely manner. Various diagnostic assays aimed to detect virus specific antibodies and infectious virus/viral component are available to determine the status of BVD virus in the herds (Sandvik, 2005). Detection of PI animals

at early stage particularly soon after birth is of significant benefit to implement BVDV control programs. Diagnostic testing is available for the detection of the virus, BVDV specific Ag, and BVDV specific Abs. These tests are generally reported as being very reliable (Saliki and Dubovi, 2004; Dubovi, 2013). Different diagnostic methods used are the following:

SEROLOGICAL METHODS

Serological methods can also be used to diagnose acute infection by detection of humoral immune response with follow up re–sampling. For the detection of sero–conversion, various serological assays have been used for BVD virus. Among these, serum neutralisation and enzyme linked immunosorbent assays (ELISA) are considered more sensitive.

SERUM NEUTRALIZATION TEST (SNT)

Serum Neutralization (SN) test is taken as gold standard for antibody titration. It is specific and sensitive, but due to involvement of cell culture is labour demanding and will take 5–6 days to perform. Thus, it is usually used as a for back–up test for reference (Sandvik, 2005). The antibodies detected are mainly against E2 protein of virus and antibody titre in the same sample may vary depending upon the strain of virus used in the assay (Jones et al., 2001; Couvreur et al., 2002). Cytopathogenic strains (Oregon C24V and NADL) of BVD virus are usually used for titration of antibodies. Now immune conjugates based assays are available that permit detection of neutralizing antibodies against non–cytopathic biotype of viruses. Pooled samples for determination of antibodies level against BVD virus can give indication about the status of BVDV in a herd (Niskanen, et al., 1991; Niskanen, 1993; Houe et al., 1995; Paton et al., 1998; Lindberg and Alenius, 1999; Pritchard, 2001; Valle et al., 2005). Bock et al., (1997) determined the proportion and incidence of PI calves with pestivirus in Australian herds. Serum neutralization (SN) and an antigen–capture ELISA (AC–ELISA) tests were applied to determine antibody and antigen to bovine pestivirus respectively. The calves were also examined for pestivirus by inoculating pooled lymphocyte samples from calves in the sheep. The study included eight herds. Serum neutralization test was used as screening test and antigen–capture ELISA as follow up test. The animals having SN antibody titers ‹ 1: 32 were further processed for confirmation of pestivirus antigen. Out of total 1521 animals, 0.9% (14) was found PI with an incidence ranging from 0.0 to 3.0 % per year over 6 years. In the study, off eight test herds, 04 were found with PI animals. Based on the findings, it could be concluded that sheep inoculation, paired AC–ELISA and SN tests in combination can be used for detecting persistently infected calves with bovine pestivirus with highly sensitivity and specificity. In another study, virus neutralization test was used to measure the neutralizing antibodies to genotype 1 and 2 of bovine viral diarrhea virus using cell culture. The presence of antibodies can be confirmed by inhibition of viral cytopathology or by immunoperoxidase staining for cytopathic and noncytopathic strains respectively. Monoclonal antibody $15C_5$ specific for BVD virus, biotinylated rabbit anti–mouse antibody, horse reddish peroxidase–streptavidin and 3–amino–9–ethyl carbazole as substrate was used. Twenty strains of BVDV consisting of 14 of type 1 and 6 of type 2 were used to infect cells in the lab. The serum containing antibodies against both type 1 and 2 was used as positive control serum. Regardless of biotype, no significant differences in antibody titers for respective type strains, was observed. It was also found that calves vaccinated with either modified live virus or inactivated vaccine (BVDV type 1) depicted higher antibody response to type 1 strain compared to type 2 strains. Thus, although, the genotypes are differentiated by non–coding sequences, there appears to be more vigorous virus neutralizing Ab response by genotype homologous antibody (Fulton et al., 1997).

ANTIBODY CAPTURE ELISA

The ELISA test is advantageous by SNT for being rapid, relatively inexpensive, and easy to establish and run. Large number of samples can be processed within short time. Two different ELISA formats are in use to determine the antibody status of the herd: indirect or blocking (competitive) assays. In the indirect format, the ELISA plates are coated with viral antigen and specific antibodies are trapped by immobilized viral antigen. The specific reaction is subsequently detected using enzyme conjugated species–specific anti–antibodies. A positive reaction is interpreted reading the optical density (OD) of color which developed on addition of substrate solution. In blocking ELISAs, conjugated virus–specific antibodies binding to adsorbed antigen is blocked by virus–specific antibodies in the sample. Thus the positive sample will express no or low OD relative to negative reference serum.

DETECTION OF BVDV

In principle, three classes of methods like detection of virus, its nucleic acid and virus isolation, are in use. Blood, serum, faces and skin biopsies of infected animals can be used for detection of BVD virus and viral genome (Sandvik et al., 1997a; Bruschke et al., 1998; Ellis et al., 1998). From persistently infected animals, BVDV antigen can be detected throughout their life. Commonly used methods include virus isolation, different immune based antigen detection assays, such as ELISA or immunohistochemistry (IHC), and reverse transcriptase–polymerase chain reaction (RT–PCR). Virus isolation and AC–ELISA, however may be negatively influenced by maternal antibody, while, IHC and PCR have proved to be effective even in the presence of antibodies (Zimmer et al., 2004; Kuhne et al., 2005; Njaa et al., 2000; Horner et al., 1995).

VIRUS ISOLATION (VI)

BVDV was first isolated as a cytopathogenic agent in bovine kidneys cell cultures (Underdahl et al., 1957). BVD virus has been isolated in numerous types of bovine cell cultures such as bovine fetal kidney, bovine turbinate cells, bovine testicular cells, Madin Darby Bovine Kidney (MDBK) and bovine endothelial cells (Sandvik, 2005; Cornish et al., 2005). BVD virus is relatively easy to isolate in cell cultures. CP strains of BVDV induce cytopathic changes on cultured cells within 48 hours post inoculation. However, generally, BVD field virus isolates are non–cytopathic. Virus isolation using bovine cell cultures, followed by confirmation through immunoperoxidase or immunofluorescence staining is virus isolation (VI) in bovine cell cultures, is considered to be the standard test (Meyling, 1984). For confirmation of NCP strains, usually 3 to 5 days are required. Serum, blood, nasal

swabs, semen and tissues samples may be used for diagnosis of BVD virus. White blood cells are most commonly used for screening of neonatal calves but use of VI test in neonatal calves is not dependable due to the presence of passively derived maternal antibodies, or cytotoxic sera, both of which can yield false negative results (Bolin et al., 1991). Moreover, it is compulsory that fresh cell cultures must tested before use to rule out any viral contaminants (Bolin et al., 1994; Edwards, 1993). Liquid nitrogen can be used to preserve primary or secondary cultures in frozen form. Bovine viral diarrhea virus free cell lines can be maintained by the use of continuous cell line through regular testing (Bolin et al., 1994). The fetal bovine serum used to supplement the cell culture should be free from both BVDV and its neutralising antibody (Edwards, 1993). Destruction of BVDV in serum by irradiation at 25 kiloGrays (2.5 Mrad) is more reliable than that of heat treatment at 56°C for 30–45 minutes. However, irradicated commercial batches of fetal bovine serum remained positive by PCR. Where suitable, bovine fetal serum can be replaced by horse serum. Buffy coat, whole blood, leukocytes or serum are suitable for isolation of the virus. Maternal antibodies may interfere virus isolation in case of serum samples. Therefore procedures for virus isolation should be optimized to give maximum sensitivity.

ANTIGEN CAPTURE ELISA (AC–ELISA)

Several formats of ELISA are commercially available for detection of viral antigens. The AC–ELISA is mostly based on MAb specific to viral antigens (Fenton et al., 1991; Mignon et al., 1992; Shannon et al., 1993; Shannon et al., 1991). The basic principle is based on the use of virus–specific monoclonal antibodies reaction with capture viral antigens and its detection by enzyme–conjugated antibodies. Antigen capture ELISA is widely used for identification of PI animals, and can be used for detection of virus in serum, buffy coat cells or skin biopsies (e.g. ear notch samples). Antigen capture ELISA may yield false negative results if antibodies are present in the sample. This should be considered when testing blood based diagnosis in young animals that might have persisting maternal antibodies (Zimmer et al., 2004). In a study conducted by Mignon et al., (1992), Bovine viral diarrhea virus was detected in blood samples by an enzyme–linked immunosorbent assay (ELISA). A total of 761 samples of known status (viraemic or not) were evaluated. The sensitivity, specificity and predictive values of the assay were 100% compared to that of virus isolation (90%). ELISA was proven good replacement of virus isolation techniques for detection of BVD virus in persistent animals. In another study, antigen–capture ELISA (AC–ELISA) was used to detect pestivirus in persistently infected cattle. Various samples like blood clots, blood leukocytes and tissue samples were tested in this study. A complete agreement was found between ELISA and conventional virus isolation procedures. Three broadly–reactive monoclonal antibodies were used to detect captured antigen. Higher optical densities for blood clots and blood leukocytes from infected animals were observed than uninfected animals. Spleen and liver samples of carrier cattle had OD values of 1.77 and 0.95 respectively with ‹ 0.20 for negative tissue samples. The AC–ELISA was found to be suitable for regular diagnostic and certification testing (Shannon et al., 1991). Fulton et al., (2006) evaluated the efficacy of vaccine by challenge study using noncytopathogenic BVDV2a. Various tests were also compared to discriminate BVDV transiently infected calves from PI calves. Ear notches were collected from persistent and transiently infected animals. Fresh notches were tested through an antigen–capture enzyme–linked immunosorbent assay and formalinized by immunohistochemistry test to detect BVDV antigen. Both assays failed to discriminate persistent animals from transiently infected animals. In another study, for the detection of BVD virus, 860 blood samples without antibodies were tested through both virus isolation and in an antigen–capture enzyme linked immunosorbent assay (ELISA) based on monoclonal antibodies (MAbs) against the nonstructural BVD virus protein p125/p80. A total of 843 samples (98%) were positive (n= 170, 20%) or negative (n = 673, 78%) in both tests, corresponding to an agreement of K = 0.94. Among 17 samples with diverging results, 3 were from animals transiently infected with BVD virus, and 5 came from clinically affected animals. The reactivity of the MAbs was controlled against 387 field isolates of BVD virus. All were detected by the MAbs, thereby confirming the general view that the p125 virus protein is highly conserved among different BVD viruses (Sandvik and Krogsrud, 1995). Kuhne and colleagues applied an antigen capture enzyme linked immunosorbent assay on ear notch biopsies from cattle to detect bovine viral diarrhoea virus (BVDV). After processing a total of 99 BVDV positive and 469 negative samples, a sensitivity of 100% and specificity of 99.6% was found. It was also found that after intake of colostrums, positive serum samples turned negative while ear notch biopsies remained positive all the times for BVDV. Testing multiple ear samples from PI cattle yielded consistently positive results. The author concluded that, ear samples testing through ELISA could be used as a reliable and economic way of BVDV testing (Kuhne et al., 2005). Efficay of 2 commercial antigen capture enzyme linked immunosorbent assays to detect bovine viral diarrhoea virus (BVDV) in serum and skin biopsies.was evaluated by Hill et al., (2007). Ear notch biopsies and serum samples were collected from 30 known persistently infected cattle and 246 cohorts as well. Skin biopsies elutes were collected after soaking overnight in buffer. Both elutes and sera were tested through two commercially available ELISAs for detection of BVDV antigen. Furthermore, to validate the results of ELISAs, a subsample of positive and negative sera was also tested using a polymerase chain reaction (PCR) test. A study was also undertaken to determine the possibility of cross contamination that may occur during collection and processing of skin tissues. All the samples which were found positive for persistent infection through either ELISA remained positive by PCR showing a perfect agreement between all assays. No evidence of cross–contamination during collection and processing of skin samples was observed in this study.

IMMUNOHISTOCHEMICAL ASSAYS

In the recent years, a new technique “immunohistochemistry (IHC)” for the detection of BVD virus using skin biopsies had been introduced earlier by Thur et al., (1996).

Njaa et al., (2000) detected positive staining in 41 of 42 formalin–fixed, paraffin–embedded skin samples from

persistently infected calves using peroxidase based IHC technique. The ear skin biopsy is now being used to screen herds for persistently infected cattle particularly for screening of young calves due to relative ease in collection of sample and independence from risk of interference with persistent maternal antibodies (Brodersen, 2004). Driskell and Ridpath, (2006) assessed current BVDV detection methods being used at various laboratories in USA. Data from 26 veterinary diagnostic laboratories in 23 states was collected which revealed no clear consensus on BVDV testing method. Further, it is found that that ear–notch antigen capture enzyme–linked immunosorbent assay (ACE) was the test most commonly used test for the detection of BVDV. Groom and Keilen, 2002 evaluated the use of peroxidase based immunohistochemical staining (IP–IHC) for early detection of persistent BVDV infection using skin biopsy samples from neonatal calves. A total of 332, 1 to 4–week–old dairy calves were screened for BVDV. Immunohistochemistry (IHC) staining results for BVDV antigen on formalin–fixed skin biopsy samples were compared to those of virus isolation (VI) from white blood cell preparations. Six calves were taken as persistently infected with BVDV by both IHC and VI tests. Virus isolation detected one acutely infected calf which was found negative by IHC. However, on follow up test, the calf was tested negative by VI. Thus, immunohistochemical staining of skin biopsy samples was found a reliable and useful management tool recommended as in aid of controlling and preventing BVDV infection. Cornish et al., (2005) compared immunohistochemistry (AP–IHC) and antigen–capture ELISA (Ag ELISA) on ear notches, for detection of BVDV persistent infection (PI) in 559 Angus calves aging from 1 and 5 months. Virus isolation and reverse transcription (RT–PCR) tests on buffy coat for detection of BVDV infection were also applied. Serum neutralization (SN) test was used to determine level of antibodies to BVDV types 1a and 2. A total of 67 out of 559 (12.0%) calves tested positive at initial screening by IHC using alkaline phosphatase system, Ag ELISA, or VI tests. All positive calves were kept for a minimum of 3 months for repeat testing monthly by IHC, Ag ELISA, VI, RT–PCR, and SN. Of these calves which were positive at initial screening, 59/67 (88.1%) were found PI and 8/67 (11.9%) acutely infected. Both IHC and Ag ELISA detected 100% of PI calves. In the study however, IHC and Ag ELISA also detected 6 and 8 acutely infected calves, respectively, at initial screening. Furthermore, IHC and Ag ELISA continued to detect acutely infected calves 3 months after initial screening. Indistinguishable IHC staining signals from PI calves, in 3 acutely infected calves were observed at initial screening. It is recommended that, both IHC (IP–IHC) and Ag ELISA were accurate in detecting PI animals but both tests also detect some calves acutely infected with BVDV due to which, repeat testing using VI or RT–PCR on buffy coat samples was suggested, usually at 30 days after initial screening to conclusively distinguish between acute and PI. Luzzago et al., (2006) evaluated the reliability and feasibility of IHC using immunoperoxidase label (IP) on ear skin tissues to detect PI animals in field conditions, including both adult and calves less than 6 months of age. In animals over 6 months of age, skin biopsy and blood sample were collected at the same time, whereas in young calves blood sampling was performed when animals reached 6 months of age. One hundred and sixty–five animals were tested, and immunohistochemical results were compared with those of antigen ELISA. In case of inconclusive results, virus isolation and virus neutralization assays were performed. Agreement K value was 0, 96. Immunohistochemical staining in positive animals was clearly detectable in the keratinocytes of the epidermis and adnexa. The author concluded that, IP–IHC on skin biopsies is a reliable test for identification of PI animals, and provides an alternative and/or complementary method to VI and antigen ELISA, particularly in neonatal calves, where the sensitivity of the latter tests can be hampered by the presence of maternal antibodies. In addition fixed tissues did not present the inconvenience of laboratory virus contamination. Provided that prolonged fixation was avoided, IHC was an inexpensive, sensitive, specific and reliable diagnostic test to identify persistently infected cattle. Baszler et al., (1995) processed 50 formalin–fixed paraffin–embedded tissues from spontaneous cases (39 bovine, nine ovine, two caprine) of bovine viral diarrhea virus (BVDV) infection by virus isolation and alkaline phosphates based immunohistochemistry (IHC) using anti–BVDV gp–43 monoclonal antibody (Mab 15C5). In the study, virus isolation and IHC was compared in determining BVDV and cellular distribution of BVDV in various clinical manifestations of infection. In bovids with abortion enteric (mucosal disease, acute and chronic diarrhea, neonatal diarrhea) and respiratory disease, 100% concordance of virus isolation and immunohistochemistry was found. When laboratory tests applied on gastrointestinal tissue and/or feces, immunohistochemistry detected 100% BVDV cases whereas, virus isolation detected BVDV in only 65% of cattle. In all clinical forms of BVDV infection, distribution of BVD virus was widespread in various tissues of individual cattle. In the absence of other pathogens, viral antigen accumulation was correlated with tissue only in the lung, placenta gastrointestinal tract, lymphoid tissue and eye. This study demonstrated the usefulness of immunohistochemistry to diagnose BVDV infections in cattle. Hilbe et al., (2007) compared five diagnostic tests (peroxidase based immunohistochemistry (IP–IHC), 2 commercial antigen ELISAs, 1 commercial antibody ELISA, and real–time RT–PCR) for the detection of bovine viral diarrhea virus infection using skin biopsies (shoulder region) and/or serum. A total of 224 calves (0–3 months of age), 23 calves (›3 months but ‹ 7 months) and 11 cattle (› 7 months) were included in the study. Both skin and serum samples were found equally appropriate by 3 antigen detection methods and the real–time RT–PCR. Off 249 samples, 26 were BVDV–positive with all antigen detection methods and the real–time RT–PCR while 9 out of 258 samples with discordant results were retested by RT–PCR, RT–PCR reamplification (ReA), and antigen ELISA I on serum. Immunohistochemistry on formalin fixed and paraffin–embedded skin biopsies was also performed. These discordant samples were also processed for virus isolation and subsequently for genotyping. Transiently infected animals were identified in 3 cases while 2 samples which were tested positive by real–time RT–PCR were recognized false positive due to cross–contamination. Due to the presence of maternal antibodies, the antigen ELISA II failed to detect 2 BVDV–positive calves. The cause of false–positive results in this ELISA remained uncertain. The

author concluded that, only IHC (IP) or antigen ELISA I assays on skin samples can be efficiently used to detect persistently infected animals. Thur et al., (1997) demonstrated BVDV in fetuses by peroxidase–immunohistochemical (IP–IHC) methods on cryostat and paraffin sections, by virus isolation in cell culture and in some instances, an antigen capture ELISA. Immunohistochemical methods and virus isolation in cell culture sensitivity for detection of BVD virus was equal; nevertheless, it decreased during autolysis. In such cases, use of paraffin–embedded, formalin–fixed brain sections was the most suitable method whereas; antigen detection by ELISA was less sensitive. In this study, it is concluded that immunohistochemical analysis of cryostat sections of thyroid gland, brain, skin, placenta and abomasum, is a fast, sensitive method for detecting pestiviruses in fetuses. Formalin–fixed, paraffin–embedded brain sections were mostly recommended among other described methods in the presence of advanced autolytic changes.

POLYMERASE CHAIN REACTION (PCR)

Reverse transcription–polymerase chain reaction (RT–PCR) is a quick and sensitive technique for detection of viral RNA. In the conventional PCR protocols, various steps (extraction of RNA, reverse transcription to cDNA, amplification and detection of amplicons) are carried out separately, which is time–consuming. The necessity of opening the PCR tube for product detection increases the risk of false positive results due to amplicon contamination.

More recent real–time RT–PCR systems minimize these drawbacks, as after RNA extraction, all steps are carried out in a single tube thus eliminating the risk of carry–over contamination (McGoldrick et al., 1999). The Real time PCR assays are excellent tools for rapid identification of viral nucleic acids, mutation analysis, genotyping of various field isolates, studying viral load and epidemiology (Ginzinger, 2002; Mackay et al., 2002). Simultaneous quantification, detection and genotypes of causative agents can be acomplished by the use specific primers and probes in the same assay (Letellier & Kerkhofs, 2003). In these assays, the quantification is done by determining the cycle threshold (Ct value) through real time fluorescence monitoring during the exponential growth phase of PCR reactions (Mackay et al., 2002; Ong and Irvine, 2002). Ct value is taken as the PCR cycle at which the product specific fluorescent signal is significantly higher than the average background signal. It is actually the point at which PCR amplification enters the exponential phase. Various chemistries to generate the fluorescent signals are being used. These chemistries can be, sequence independent or sequence specific. The sequence independent dyes as SYBER Green1, YOPRO–1, ethidium bromide, Thiazole orange, yellow orange, and Enhan CE bind to ds DNA molecules and emit fluorescence upon excitation and do not bind with ss DNA. (Garcia–Canas et al., 2002; Ginzinger, 2002; Mackay et al., 2002) Among these dyes, SYBER Green1 is perhaps the most widely used. It is a minor groove binding dye (Bustin, 2000; Mackay et al., 2002). The major disadvantage is its non–specific binding to any double–stranded DNA, including primer dimers and non–specific products, so specificity is determined only by specific primers (Bustin, 2000; Skeidsvoll and Ueland, 1995). A melting curve analysis is needed to be performed at the end of the reaction to differentiate specific signals from non–specific signals (Bustin, 2000; Mackay et al., 2002). In contrast to SYBER Green 1, sequence specific chemistry is based on the ability of confirmatory probes(s) with the fluorescent label(s) to bind its complementary sequence on one or both strands of the target DNA. These formats include TaqMan (Hydrolysis) probes, displaceable beacons, cleavable beacons and Amplifluor Uniprimer system (Bustin, 2000; Ginzinger, 2002; Mackay et al., 2002). TaqMan chemistry is based on the ability of 5' to 3' nuclease activity of Taq or Tth DNA polymerase to generate a fluorescent signal by the cleavage of fluorescent reporter at the 5' end of the probe when it hybridized to its complementary sequence (Bustin, 2000; Mackay et al., 2002). TaqMan probes are also called hydrolysis probe because of the fact that they are hydrolyzed by the nuclease activity of the enzyme. Currently the most popular real–time PCR assay principle is based on the binding of a dual–labelled probe to the PCR amplicon and the release of a signal by loss of fluorescence quenching as chain reaction degrades the probe. The dual–labelled probes used in real time PCR are designed in such a way that they have 5 to 10 C higher melting temperature (Tm) than the two primers. This allows the probe to remain bound to its target strand during the primer extension (Bustin, 2000; Ginzinger, 2002). In the recent years, there has been an increasing interest in the use of real time PCR for detection of BVDV and other important viruses. Horner et al., (1995) evaluated the suitability of three different tests for the confirmation of ruminant pestivirus infections. Reference strains of bovine viral diarrhoea virus (BVDV) and buffy coat samples from persistently infected (PI) carriers were used for sensitivity studies. Reverse transcription– polymerase chain reaction (RT–PCR) was found with greater sensitivity than the other tests. Furthermore, the antigen capture enzyme–linked immnunosorbent assay (ELISA) due to least sensitivity could only be used on tissue or blood samples. In the study conducted on clinical samples, the RT–PCR detected the most positives (42/169) compared to the ELISA (32) and the immunoperoxidase test (IPT) (20). The RT–PCR was found successful even in the presence of specific antibody in the sample. The poor sensitivity of the IPT was related to testing of toxic or contaminated or the use of a 1 passage (4–day) test and the samples. For large scale testing for diagnosis and control of pestivirus infections, ELISA was found to be most suitable assay to be used. Bhudevi and Weinstock (2003) identified BVD virus in freshly processed formalin–fixed paraffin embedded tissue sections and archival samples from both acutely and persistently infected animals up to 7 years old by real time quantitative RT–PCR using TaqMan probes. To see the effect of RNA degradation due to tissue processing and handling, fresh tissue biopsies from a BVDV infected persistent calves were stored at 4°C or room temperature for up to 7 days before formalin fixation for 24 hours and histologic processing. Samples which were stored at 4°C for 7 days prior to fixation were positive while samples kept at room temperature remained positive at 74 hours but turned negative after 96 hours. Mild decrease in signal strength was observed in fresh tissue fixed in formalin for 1 week prior to processing compared with tissue fixed for 24–48 hours. Real time RT–PCR improved diagnosis of BVD infection by allowing

prospective and retrospective identification of BVD virus in tissues. Kennedy et al., (2006) conducted a study to detect BVDV persistently infected (PI) animals using ear notch samples. Peroxidase based immunohistochemistry (IP–IHC), reverse transcription–polymerase chain reaction (RT–PCR) and individual antigen–capture enzyme–linked immmunosorbent assay (AC–ELISA) on pooled supernatants of ear–notch were compared with samples from 3,016 heifers. Individual AC–ELISA tests were compared with RT–PCR ear–notch pools with sampldes from all 3,599 heifers. Only four heifers were tested positive by both AC–ELISA and IHC. When RT–PCR was applied on each of randomly pooled ear notch supernatant from 100 animals, 2 pools were identified that contained one positive AC–ELISA sample and 1 pool that contained two positive AC–ELISA samples. Furthermore, pooled RT–PCR ear notch supernatant detected 100% (n 5 36) samples which were spiked with supernatant from selected positive AC–ELISA ear notch. Though repeat confirmatory tests were not completed, all 3 methods showed perfect agreement (100%) in detecting suspected PI animals (kappa value of 1). The application of RT–PCR on pooled ear–notch supernatant could be a good choice which is rapid, cost–effective for initial screening of cattle herds for BVDV PI animals. Subsequent testing of individual samples in positive pool by an AC–ELISA could minimize the risk of virus exposure to other animals due to rapid test results. Ridpath and Bolin (1998) used polymerase chain reaction (PCR) for classifying BVDV isolates into genotypes and subgenotypes, CSVF, BDV, BVDV1a, BVDV1b and BVDV2 on the basis of 5' un–translated region sequences. A total of 345 previously classified viral isolates from cattle and small ruminants were used to validate differential PCR tests. A perfect agreement (100%) was found between classification by differential PCR and the previous segregation of these viral isolates. Ridpath et al., (2002) studied the ability of polymerase chain reaction amplification followed by probe hybridization (RT–PCR/probe) of serum samples to detect PI animals and peroxidase–immunohistochemical for viral antigen in skin biopsies (IHC) to detect acute BVDV infections. A total of 16 BVD virus and antibody free, colostrum– calves were challenged with 6 different BVDV strains. Virus was detected 19% acutely infected animals by the RT–PCR/probe technique while no acutely infected animals were tested positive by IHC. Mahlum et al., (2002) stated that polymerase chain reaction (RT–PCR) is fast and more sensitive compared to cell culture isolation; however test results can be compromised by sample contamination during nucleic acid amplification. In this study a closed–tube format of BVDV nucleic acid amplification and detection by TaqMan RT–PCR was used and results were compared with those of virus isolation, IPMA, and IP–IHC. TaqMan RT–PCR detected BVDV in many samples which were tested negative by IPMA, IHC, and virus isolation. Only one sample was found was positive by IHC. The study revealed that TaqMan RT–PCR in a closed–tube is a rapid, economical and sensitive method to be used for BVDV detection without concerns of amplified cDNA product contamination. Baxi et al., (2006) detected and classified bovine viral diarrhea viruses (BVDV) by one–step multiplex real–time reverse transcriptase–polymerase chain reaction (RT–PCR) using SmartCycler technology and TaqMan probes. Common primers and type specific TaqMan probes for genotype 1 and 2 of BVDV were designed in the 5'–untranslated region of the viral genome. The detection limit of real–time assay was found to be 10–100 TCID50 of virus, with correlation coefficient (r2) values of 0.998 and 0.999 for BVDV1 and BVDV2, respectively. The probes were found highly specific, no reactivity with the closely related pestiviruses, classical swine fever virus and border disease virus was observed. The assay accurately classified 54 BVDV strains and field isolates with high reproducibility. There was a full agreement between one–step real–time RT–PCR assay and virus isolation for bovine serum samples. One–step real–time RT–PCR assay appears to be a rapid, sensitive, and specific test for detection and typing of BVDV. Drew et al., (1999) used a single step, single–tube reverse transcriptase–polymerase chain reaction (RT–PCR) to detect bovine viral diarrhoea virus (BVDV) in somatic cells from bulk milk samples. Samples from 80 herds with a history of BVDV were tested to validate the assay and the findings were compared with those of samples originating from same sized control group. A total of 20.5% of herds with a history of BVDV were found positive while all were found negative in control group. The assay proved specific and sensitive. It detected one persistently infected (PI) animal out of 162 lactating animal herd. On follow–up blood testing from 19 herds by RT–PCR, ten herds were positive containing at least one lactating PI animal. The authors concluded that for control strategy aiming detection and culling of PI lactating cattle at the time of sampling, the test provides a rapid and inexpensive alternative to individual animal testing for cows.

GENOTYPING

The genetic typing of BVDV has most frequently been based on sequence analysis of the 5' NCR, Npro or E2 regions (Vilcek, et al., 2001; Becher, et al., 2003; Nagai et al., 2004; Toplak et al., 2004). Analysis of the 5' NCR, a highly conserved region of the genome, has shown to be a reliable and reproducible method for genetic characterization of BVDV isolates (Ridpath, 2005b). Furthermore, it is the target region for most PCR–based diagnostics, and as such a suitable target for direct sequencing from the PCR product. Inspite of the presence of type 2 of BVD virus, subtype 1a of genotype 1 of BVD virus is predominant in UK herds. On the basis of phylogenetic analysis of viral genome at 5' untranslated region, subtype 2a of BVD virus was recognized and this was similar to that of low virulent US strain of type 2 of BVD virus which was also verified by monoclonal antibodies (Wakeley et al., 2004). Reverse transcription–polymerase chain reaction (RT–PCR) was used to identify BVD virus from diarrheal stools, intestine and bovine abortuses. The positive samples were also tested by virus isolation. The positive samples were sequenced on 5'UTR and analyzed. A total of 4 viruses (two bovine abortuses, one intestine, and one diarrheal stool) were isolated.by RT–PCR.One BVD virus isolated from bovine abortuses was biotyped as cytopathic and all other 3 were accepted as non–cytopathic. Out of 4 isolates, 3 were of genotype 1 and one diarrheal stool isolate was identified as type 2 of BVD virus. Furthermore, the type 2 of BVDV showed more similarity with that of found in North American strains than Asian strains (Park et al., 2004). Single tube TaqMan based RT–PCR assay was used to classify BVD virus into genotypes. Bovine viral diarrhea

virus was quantified by ABI PRISM 7700 sequence detection system and 2 flourogenic probes for 5' UTR. Seial 10 fold dilutions of RNA were made and sensitivity of the assay was established and compared with standard RT–PCR and 2 tubes TaqMan assay. Single tube assay was found 10 to 100 times more sensitive than 2 tube TaqMan assay and standard RT–PCR. The single tube assay was also found rapid, sensitive and specific for detection, quantification and classification of BVD virus (Bhudevi and Weinstock, 2001). To evaluate the proficiency of current methods used in various diagnostic labs, for the detection of BVD virus, a total of 4 samples (2 negative, one PI and other with undetectable amount of virus in serum by virus isolation) were submitted to 23 labs. Samples submitted were serum for AC–ELISA, RT–PCR and VI, whole blood for RT–PCR, VI, skin of ELISA and IHC. Among all the assays, AC–ELISA on skin biopsies revealed maximum uniformity in detecting positive among labs. RT–PCR and IHC correctly identified around 85% BVDV positive samples while VI using serum showed poor consistency and lowest level of agreement. The finding of this study suggested a need for standardization of test methods (Edmondson et al., 2007).

CONCLUSION

Ear notch and serum could be used for the diagnosis of PI animals using Real time RT–PCR. All the four diagnostic approaches applied had the same specificity, but Real time RT–PCR was found to be more sensitive as compared to AC–ELISA. However, AC–ELISA still has enough sensitivity to detect all PI animals. In this study, AC–ELISA was found most cost effective following by IHC, Real time RT–PCR and VI. AC–ELISA could be used for large scale testing of PI animals, and Real time RT–PCR could be used as a follow up test for suspicious samples to make the diagnosis cost effective.

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