



RESEARCH ARTICLE

Comparative Suitability of Ear Notch Biopsy and Serum Pairs for Detecting Nature of Bovine Viral Diarrhoea Virus Infection in Dairy Herds

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ABSTRACT

Suitability of ear notch biopsy (EN) and serum pairs (n= 307) collected from 10 Holstein dairy herds located in Charlottetown, Canada was evaluated for simultaneous detection, nature of bovine viral diarrhoea virus (BVDV) infection and genotype of the prevailing BVDV through Real time RT-PCR. Depending upon vaccination status and age, the sampled animals were categorized into two groups, A (n=123, ≤ 6 month of age) and B (n=184, ≥ 6 months of age) originating from 4 vaccinated (n=108) and 3 non-vaccinated (n=76) animal herds. On first round of testing a discrepancy between ear notch biopsies and sera pairs (3.25 and 6.50%; P<0.05) of groups A was observed, however, a complete harmony (50% for EN and sera each, P<0.01) was found on second round of testing that confirmed the presence of 4 persistent infection (PI) animals harboring genotype 1 of BVDV. Complete concordance between EN and sera pairs (P<0.01) on first and follow up testing in group B was observed (2.77%, each), depicting 3 PI animals with the same genotype as in group A. In the study, ear notch biopsies did not detect any transient infection (TI) but sera samples detected 3.25% transiently infected animals in group A that was 1.30 % among all the test samples (n=307) while no TI animal was found in group B. It may be concluded that both the serum and ear notch biopsy can be used to detect PI animals and that, serum samples are more sensitive than ear notch (P < 0.05) for detection of TI using real time RT-PCR.

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INTRODUCTION

Bovine viral diarrhoea virus (BVDV) belongs to pestivirus of family Flaviviridae (Wegelt *et al.*, 2011). It is one of the most important viral pathogens of cattle with worldwide distribution (Gunn *et al.*, 2005). Additionally, this virus can also cause infection in sheep, goats, camels and swine etc. (Nettleton, 1990; Vilcek and Nettleton, 2006). BVDV is a heterogenous group of viruses which are classified into two genotypes, BVDV-1 and BVDV-2, based on the genetic make up (Becher *et al.*, 2003). Each BVDV genotype exists as a cytopathogenic (CP) and non cytopathogenic (NCP) biotype. Bovine viral diarrhoea virus may cause acute transient (TI) or persistent infection (PI) depending upon the stage of gestation and type of infecting viral strains. CP viruses have only been isolated in connection with outbreaks of mucosal disease while

NCP viruses of both genotypes are commonly found in nature and cause PI in animals by avoiding the induction of a type I interferon response in the fetus and establishing immune tolerance at the time when fetus differentiate “self” from “non-self” (Charleston *et al.*, 2001; Peterhans *et al.*, 2010). The economic losses due to infection have been noted due to transplacental infection leading to reproductive failures, still birth, mummification, abortion, persistency and secondary infections by other pathogens (Ackermann and Engels, 2006). In many countries, control programs with vaccination and without vaccination are being applied for the eradication of the diseases of economic importance; however, success of the programs depends on the ability to truly detect all PI animals at a young age which are main source of the infection within herds.

Currently most diagnostic laboratories are offering an array of diagnostic tests to detect BVDV infected animals such as virus isolation (VI), reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry (IHC), and antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) because no one test is considered perfect in all situations (Dubovi, 1996). Among these diagnostic tests, RT-PCR test is rapid, reliable and independent of the problem of colostral antibody intervention as is observed in VI and AC-ELISA for detection of persistent or transient infections in young calves (Kozasa *et al.*, 2005). Real-time PCR has the same advantages as conventional PCR assays, but is more rapid due to real time visualization of data (Mackay *et al.*, 2002). Most of the diagnostic laboratories are using different samples like serum or milk samples for detection of BVDV however in these days; ear notch biopsies are getting attention of diagnosticians as preferred samples due to ease in its collection and liberty of interference by colostral antibodies in diagnosis. Suitability of these samples is still questionable which is needed to be addressed. Present study is, therefore, designed to compare the diagnostic application of serum and ear notch biopsy pairs for simultaneous detection and genotyping of prevailing BVDV through real time RT-PCR using TaqMan probe system.

MATERIALS AND METHODS

A total of 307 ear notch biopsies and serum pairs were collected from 10 BVDV suspected Holstein dairy cattle farms in and around Charlottetown, Canada. According to vaccination history and age, the sampled animals were separated into two groups, A (n=123, ≤ 6 month of age, A1, A2, A3), B (n=184, ≥ 6 months of age) containing both vaccinated (n=108, BV1, BV2, BV3, BV4) and non-vaccinated animals (n=76, BN1, BN2, BN3). Both sample pairs were processed to compare the diagnostic suitability in simultaneous detection of BVDV status and genotyping of virus strain through real time RT-PCR. To confirm PI status of animals, all animals tested positive were re-sampled 30 days after the first round of testing and processed again through PCR.

Real time RT-PCR: Two reference virus strains of BVD virus (NADL- BVDV1, 125c -BVDV2) were processed to find out the specificity of probes before processing field samples. Both probes (TaqMan FAM and TaqMan Quasar) were tested against NADL, 125c and water (non-template control-NTC) separately. In the first reaction, TaqMan FAM probe 1 was tested against NADL, 125c and water (non-template control-NTC). In the second reaction, the same templates used in reaction first were tested with TaqMan Quasar probe 2.

Detection limit of the real time RT-PCR was determined by making serial 10 fold dilutions of reference virus strains (BVDV1-NADL and BVDV2-125c) in MEM, based on the infectious titre of the virus. Various dilutions of stock viruses ranging from 10^{-1} TCID₅₀ to 10^{-7} TCID₅₀ were made. The RNAs extractions, from each dilution, were tested through real time RT-PCR.

Extraction of total RNA from ear notch biopsies and serum samples: Total RNA from ear notch biopsies and serum samples were extracted using Qiagen RNeasy Mini Kit (QIAGEN, Cat # 74106 and 52904) according to manufacturer's recommendations. RNA was eluted with 20 μ l of RNase-free water and stored at -80°C till further use. RNA from two BVDV reference strains (BVDV1-NADL, BVDV2-125c) was also included in the assay as positive control.

Protocol for real time RT- PCR: Real time RT-PCR was performed using the primers, probes (Table 1) and protocol described by Baxi *et al.* (2006) with some modifications, using Smartcycler sequence detector (Cepheid, Inc). Qiagen quantiTect Multiplex RT-PCR Kit (Cat # 204643) was used to perform the assay. A reaction of 25 μ l volume containing 12.5 μ l of QT master mix, 1 μ l (400 nM) of each of forward and reverse primer, 0.5 μ l (200 nM) of each of the fluorescent probe (FAM-BVDV1 and QUASAR- BVDV2), 0.25 μ l of RT-mix, 4.25 μ l of RNase free water and 5 μ l of each of the extracted RNA from samples was prepared. Prior to amplification, the RNA was transcribed at 50°C for 30 min, followed by one cycle of 94°C for 15 min for activation of the HotStarTaq DNA polymerase and inactivation of reverse transcriptase. This was followed by 40 cycles of denaturation at 94°C for 15sec. and of annealing and extension at 60°C for 60 sec. The non template control (NTC) containing RNase free water and two positive controls (RNA from BVDV1-NADL and BVDV2-NVSL 125) were also processed with each reaction. Amplified PCR products were visualized in a 2% agarose gel in 1% TBE stained with ethidium bromide to confirm size of amplicons.

Statistical analysis: The data of comparative suitability of ear notch biopsy-serum pairs for detection of PI and TI animals with BVDV by real time RT-PCR was analyzed by Z-test using statistical software package STATA 9.1 (College state, Texas). P<0.05 was considered significant.

RESULTS

In the assay, amplification of PCR product is detected during each cycle by the release of a fluorescent reporter dye from a hybridization probe. The PCR products were also confirmed by gel electrophoresis (2% agarose) and staining with ethidium bromide. Test results were validated by the presence of specific curves with positive control RNA (BVDV1-NADL, BVDV2-125c) and no amplification curve with NTC. Before testing field samples, the specificity of both probes and sensitivity of real time PCR machine was determined under local conditions. The probes used were found highly specific as both TaqMan probes 1 and 2 correctly identified BVDV1-NADL and BVDV2-125 c, respectively without showing cross reactivity (Fig. 1(A, B)) while the detection limit of real time PCR machine was found to be 10^{-5} and 10^{-4} TCID₅₀ units of BVDV1 and BVDV2, respectively (Table 2). To reconfirm the probe specificity, when amplicons were subjected to electrophoresis on 2% agarose gel, a specific 107 bp sized products was observed confirming probe specificity (Fig. 2).

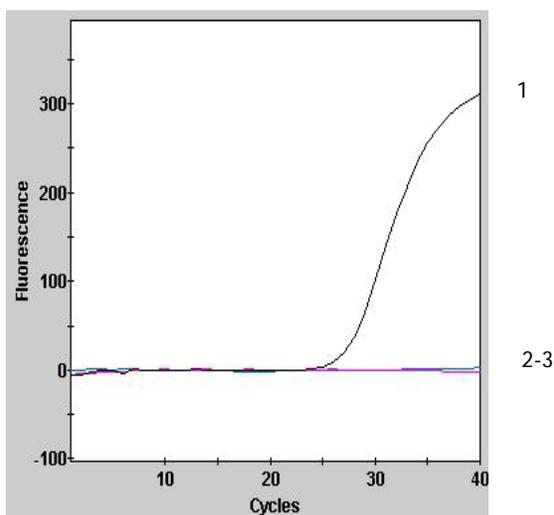


Fig. 1: Specificity of taqman probes. Quasar channel, amplification curve, 1 (I25c), 2 (NADL) and 3 (water).

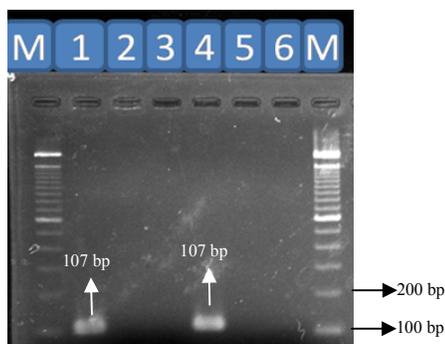


Fig. 2: Agarose gel electrophoresis of amplified products obtained by probe specificity reactions

In group A, 4/123 (3.25%) EN biopsies and 8/123 (6.50%) serum sample were tested positive, for genotype 1 of BVDV, on first round of testing (Table 3). On the other hand, out of 108 samples from vaccinated animal herds of group B, 3 RNA samples (2.77%) extracted from each of the ear notch biopsies and sera were found positive (BVDV-1) while all the animals from non-vaccinated herds of same group were established negative (Table 3). No animal of either group was found positive for genotype 2 of BVDV. To confirm the PI status of animals, the farmers were contacted again for re-sampling of positive animals. Eight animals from group A and 3 of group B were selected for re-sampling, 30 days post first sampling. The selected samples were collected and processed again following the same protocol. On second round of testing, out of 8 collected samples of group A, only 4 (50%) were found positive (BVDV-1) using both ear notch biopsies and serum samples whereas 4 serum samples which were found positive on first round of testing turned negative. Based upon the follow up testing, the results indicated the presence of 4 persistent and 4 acute transiently infected animals harboring genotype 1 of BVDV in group A.

For animals of group B, 3 animals which were found positive on first round of testing using EN and sera pairs, remained positive on follow up testing depicted the presence of 3 (2.77%) animals with PI containing same genotype as in group A (Table 3). Irrespective of

grouping, as for as the comparative suitability of both types of samples for the detection of PI and genotyping of BVDV was concerned, out of 307 tested samples, 2.28% positivity (BVDV-1) was found using ear notch RT-PCR and 3.58% (BVDV-1) by serum RT-PCR. On first round of testing, though this discrepancy was not observed on second testing, as a perfect agreement was eminent between both types of samples (Table 3). In the study, ear notch biopsies did not detect any transient infection but sera samples detected 3.25% (4/123) TI animals in group A that was 1.30% among all the test samples (n=307) while no TI animal was found in group B.

A significant difference ($P < 0.05$) between EN biopsies and serum pairs for the detection of acute transient animals with BVDV was observed while a perfect agreement ($P = 1$) for detecting PI animals by real time RT-PCR was evident in the study.

DISCUSSION

Infection with bovine viral diarrhoea virus (BVDV) may result in persistent or acute transient infection in cattle (Fulton *et al.*, 2002). Both PI and TI animals play important role in maintenance of BVD infection in dairy herds. Timely and accurate diagnosis of BVDV is of utmost importance to maintain BVDV free herds. A variety of samples like blood, bulk milk, semen and tissues etc. are used for diagnosis of BVDV with variable sensitivity. In this study diagnostic suitability of ear notch and serum pairs was established by real time RT-PCR.

Since its discovery, polymerase chain reaction (PCR) has been widely used for the diagnosis of viral diseases (Mullis *et al.*, 1994). In the protocols of classical PCR, necessity of opening the PCR tube for product detection increases the risk of false positive results due to amplicon contamination while real time RT-PCR assay used as a diagnostic tool to compare diagnostic suitability of serum and EN biopsies for the simultaneous detection of BVDV, nature of infection and genotyping of prevailing BVDV in the study area minimized the possibility of contamination. The probes used in the real time RT-PCR were found highly specific which is in line with the observation of Baxi *et al.* (2006). The detection limit of real time RT-PCR assay was found to be 10^{-5} and 10^{-4} TCID₅₀ units of BVDV1 and BVDV2, respectively (Table 2). The real time RT-PCR assay when used for processing the field samples showed a significant discrepancy in detection of

Table 1: Oligonucleotide primers and probes

PP Sequence	Position	Size
PF 5'CTAGCCATGCCCTTAGTAG3'	102	19 each
PR 5'CGTCGAACCAAGTGACGACT3'	208	
PI 5'-FAM- TAGCAACAGTGGTGAGTTCGTTGGATGGC T-BHQ3'	141	30 each
P2 5'-QUASAR- TAGCGGTAGCAGTGAGTTCGTTGGATGGC C-BHQ-3'	141	

PP-Primers and probes, PF (Pesti-forward primer), PR (Pesti-reverse primer), PI (BVDV -Probe 1), P2 (BVDV probe 2)

Table 2: Detection limits of real time RT-PCR

Genotype of Virus	Tests	TCID ₅₀						
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
BVDV1(NADL)	PCR	+	+	+	+	+	-	-
BVDV-2(125C)	PCR	+	+	+	+	-	-	-

+ = Positive reaction, - = Negative reaction

Table 3: Overall comparative suitability of ear notch biopsies and sera pairs by real time RT-PCR for the detection of BVDV infection

Group	EN Biopsies		Serum		Nature of Infection			
	1 st T	2 nd T	1 st T	2 nd T	Persistent (%)		Transient (%)	
					EN	Serum	EN	Serum
A	4/123 (3.25%)	4/08 (50%)	8/123 (6.50%)	4/08 (50%)	3.25	3.25	0.00	3.25
BV	3/108 (2.77%)	3/3 (100%)	3/108 (2.77%)	3/3 (100%)	2.77	2.77	0.00	0.00
BN	0/76	-	0/76	-	-	-	-	-
A+B	7/307 (2.28%)	7/11 (63.63%)	11/307 (3.58%)	7/11 (63.63%)	2.28	2.28	0.00	1.30

PI: Persistent infection, TI: Transient infection, 1stT= First round of testing 2ndT= Second round of testing.

BVD virus between ear notch biopsies and serum samples collected from younger animals. On first round of testing, 4 ear notch biopsies and 8 serum samples were found positive (Table: 3). On second round of testing, however, a complete agreement between ear notch biopsies and serum samples has been observed, as 4 of ear notch biopsy-serum pairs showed positive signals (Table: 3). The 4 animals out of 8, sera of which were found positive on first round of testing and turned negative on follow up testing, were acute transiently infected animals. It has been well recognized that BVD virus postnatal infected cattle herds clear the infection (Lindberg and Alenius, 1999; Viltrop *et al.*, 2002; Mainar-Jaime *et al.*, 2001; Valle *et al.*, 2005). Real time RT-PCR detected BVD virus from serum of transiently infected animals but failed to detect the virus from skin biopsies during first round of testing (Hilbe *et al.*, 2007). The animals infected with low doses of the challenge virus infection do not have sufficient amount of the virus in skin tissues. However, skin of the vast majority of acutely infected animals is also devoid of detectable amount of the virus (Ridpath *et al.*, 2002; Saliki and Dubovi, 2004).

Three out of 108 ear notch-sera pairs from animals representing more than 6 months of age, were found positive for BVD virus on first and second round of testing indicating a perfect agreement between the both types of samples (Table 3). The persistent animals had high titer of BVD virus in their circulation and skin tissues enabling its detection from both types of samples (Brock *et al.*, 1998).

On the basis of first and follow up test results, 7 animals, 4 from group A and 3 of group B, were declared as PI while 4 animals were taken as TI in younger age group A. Furthermore, all the positive animals were harbouring genotype-1 of BVDV. RT-PCR assay detected only genotype 1 of BVDV from samples of majority of PI animals (Fulton *et al.*, 2002; Uttenthal *et al.*, 2005). In the present study, genotype-2 of BVDV was not detected. However, genotype 2 of BVD is prevailing in North America, Europe, Asia and South America (Cranwell *et al.*, 2005; Barros *et al.*, 2006; Pizarro-Lucero *et al.*, 2006). However, failure of detection of genotype 2 of the BVDV in the present study could be due to limited number of test animals for genotyping.

Conclusions: In conclusion, both the serum and ear notch biopsy can be used for the detection of persistent infection and genotyping of prevailing BVDV. However, Ear notch biopsies had poor sensitivity for the detection of acute transiently infected animals in the present study.

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