

## COMPARATIVE DIAGNOSTIC APPLICATIONS OF ANTIGEN CAPTURE ELISA AND IMMUNOHISTOCHEMISTRY FOR DETECTION OF BOVINE VIRAL DIARRHEA PERSISTENT INFECTION

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### ABSTRACT

The diagnostic ability of antigen-capture enzyme-linked immunosorbent assays (AC-ELISA) and immunohistochemistry using two enzymes labels, alkaline phosphatase (AP) and peroxidase (P), to detect bovine viral diarrhoea (BVD) persistent infection (PI) was assessed using serum and ear notch biopsy pairs (n= 469) collected from 12 Holstein dairy herds located in Charlottetown, Canada. The sampled animals were divided into two age groups, A ( < 6 months, n = 146) and B ( > 6 months, n = 323). All the animals of group B were pre-screened by serum neutralization test (SNT), and those animals (n=52) which had SN titer <math>1:64</math>, as well as all ear notch biopsies (EN) of group A (n=146) were processed to confirm the BVDV persistent infection. Two EN biopsies of each groups A (1.37%) and B (3.48%) were found positive on first and follow up testing by AC-ELISA and immunohistochemical technique using AP enzyme label. Peroxidase label could not be distinguished from skin melanin, and thus was found unsuitable to differentiate between positive and negative tissue sections. There was no significant difference ( $P>0.05$ ) between AC-ELISA and IHC-AP. Real time RT-PCR validated the results as well. Genotype 1 was confirmed in the study area. The study concluded that AC-ELISA and IHC-AP were equally suitable for detection of BVDV persistent infection.

**Key words:** Prevalence, Bovine Viral Diarrhoea Virus, Persistent infection, AC-ELISA, IHC.

### INTRODUCTION

Bovine viral diarrhoea virus (BVDV) belongs to pestivirus genus of flaviviridae (Meyers and Thiel, 1996), and it is a significant viral pathogen (Gunn *et al.*, 2005) associated with reproductive, respiratory and gastrointestinal diseases of cattle (Houe, 1999). On the basis of non-coding nucleotide sequence at 5'UTR, BVDV isolates have been divided in two genotypes BVDV-1 and BVDV-2. Each genotype has two biotypes, the cytopathic (CP) and the non-cytopathic (NCP). The cytopathic biotypes have only been isolated from animals with mucosal disease, while NCP biotypes cause persistent infection in animals (Neill *et al.*, 2008).

Persistent animals have increased susceptibility to other infectious diseases, and frequently succumb to mucosal disease. Mucosal disease arises only in PI animals, either due to recombination of the BVDV genome with cellular genome, or due to horizontal superinfection with the cytopathic biotype (Brownlie *et al.*, 1984; Sandvik, 2005). Several methods have been used to screen persistent infection (Dubovi, 1996). However, in neonatal calves, presence of maternal antibodies can make the virus unavailable for virus isolation, antigen-capture enzyme-linked immunosorbent

assay and immunohistochemistry, thus false negative results with respect to BVDV persistency in blood samples can be generated. Despite the availability of a variety of diagnostic tests, BVDV is still circulating in dairy herds worldwide, and the most significant factor preventing farmers from culling of PI animals is that identification of persistently infected animals is cost prohibitive.

Antigen capture ELISA (AC-ELISA) and immunohistochemistry (IHC), specifically on ear notch biopsy specimens, have been reported as suitable methods of detection of BVDV persistency, including in young calves, because of cost effectiveness and lack of interference of maternal antibodies (Brodersen, 2004). While IHC has been recommended for detection of BVDV persistency in ear notch skin biopsy samples in herd screening, its reported sensitivity and specificity varied depending on whether AP and P enzyme labels were used (Mason *et al.*, 1986, 1991; Taylor *et al.*, 2002). On the other hand, AC-ELISA in skin biopsies has been reported as valid and economical (Kampa *et al.*, 2007).

The present study was undertaken to compare AC-ELISA and two IHC methods systems, AP and P, for the detection of BVDV persistently infected animals in ear notch sample.

## MATERIALS AND METHODS

Ear notch biopsies and serum pairs (n=469) were collected from 12 Holstein dairy cattle farms in Prince Edward Island, Canada. The sampled animals were divided into two age groups, A (n=146) originating from 04 herds (6 month of age) and group B (n=323) animals representing 8 herds (6 months of age). All ear notch biopsies of group A (n=146) and only those of group B (n=52) having SN titre 1:64 were processed to confirm the BVDV persistent infection through AC-ELISA and IHC using two labeling systems, AP and P.

Each ear notch biopsy of group A and B was divided into three parts designated as EN1a, EN2a and EN3a and EN1b, EN2b EN3b respectively for further processing. Each of EN1a, EN1b and EN2a, EN2b were processed through AC-ELISA and IHC, respectively, while EN 3a and 3b biopsies were kept as retention samples. All the animals tested positive were re-sampled 30 days after the first round of testing to differentiate transient and persistently infected animals. The animals found positive for persistent infection were processed for determination of prevailing genotype of BVDV by Real-time RT-PCR.

**Antigen Capture ELISA (AC-ELISA):** Commercial antigen detection test kit (HerdChek IDEXX, USA) was used as per manufacturer recommendations along with positive and negative controls. The results were interpreted by sample to positive (S/P) ratio of each sample, which was as negative if SP was 0.2 and 0.39 was considered as positive. A S/P ratio between 0.2 to 0.39 was considered as suspected. The suspected samples were tested twice and the S/P ratio > 0.2 was taken as positive while < 0.2 as negative.

**Immunohistochemistry (IHC):** Ear notch biopsy specimens were processed for immunohistochemical staining as described by Cornish *et al.*, 2005, with some modifications. Briefly, each of 146 EN2a of group A and 52 EN2b of group B was immersed in 10% neutral formalin for 24 hours. Paraffin blocks of formalinized biopsies were made. Briefly the tissue samples were placed in 6-chambered tissue cassettes and immersed in formalin for 1 hour and then automatically transferred in each of the two changes of 70, 90, 100% ethanol and clear rite (xylene substitute) for 1 hour each. In the final step, the tissue samples were rinsed in four changes of paraffin wax for 40 min each. The samples were paraffinized using tissue embedding machine (Tissue – TEK, TEC 5 EMA-1, Sakura Finetek, Torrance, CA). Each of the paraffinized tissue biopsies were cut at 5 µm, in quadruplicate, two of each sections were mounted on two superfrost/ plus slides (cat #12-550-15, Fisher Sci,

USA), dried overnight at room temperature, and then incubated at 60°C for 30 min. Two IHC kits, Ultra View™ Universal DAB (peroxidase-3,3 diaminobenzidine, cat #760-500, Ventana Medical Systems, Inc., USA) and Universal Alkaline Phosphatase Red detection kits (alkaline phosphatase-naphthol cat # 760-501 Ventana Medical Systems, Inc., USA) were procured for immunostaining. Tissue sections, processed with peroxidase-DAB detection kit, were designated as EN2ap and EN2bp and those, processed with alkaline phosphatase-naphthol kit as EN2aa and EN2ba. The slides were barcoded and stained (Ventana Bench Mark LT IHC staining system, Ventana Medical Systems, Inc., USA). Mounted tissues sections were deparaffinized with Ez-Prep solution. (Ventana 950-102) and processed in an automated immunostainer. The tissue sections were then treated with protease K2 at 37°C for 16 minutes for retrieval of the antigen. The sections were incubated with BVDV monoclonal antibody 15C5 (kindly donated by Syracuse Bioanalytical Inc., Ithaca, NY) at a dilution of 1:1000 in antibody diluent (Dako, North America, Inc Ref: S0809) for 2 hour. Following washing with Ventana Reaction buffer (Ventana 950-300), coverslips were applied during incubation steps to avoid evaporation. The primary antibody - alkaline phosphatase labeled antibody complexes (ENa) were visualized using Fast Red/Naphthol chromogen, while horse reddish peroxidase labeled antibody complexes (ENd) were visualized using 3,3'-diaminobenzidine.tetrahydrochloride (DAB) chromogen. Tissue sections were counter stained with Hematoxylin (Ventana 760-2021) for 4 minutes followed by 4 minutes in Bluing Reagent (Ventana 760-2037). After staining, the tissues section were dried and examined under microscope for the detection of BVDV antigen. Observation of red and brown precipitates in the epidermal, follicular and hair bulb cells were considered positive for BVDV antigen in case of alkaline phosphatase-naphthol and peroxidase-DAB detection systems, respectively.

**Real Time RT-PCR:** To validate the results of IHC and ELISA on EN biopsies, all collected serum samples of group A (n=146) and B (n=323) irrespective of SN antibody status along with AC-ELISA and IHC positive EN biopsies (n=4) were subjected to Real time RT-PCR using the primers, probes and protocol, described by Baxi *et al.*, 2006 with some modifications, using Smartcycler sequence detector (Cepheid, Inc).

**Statistical Analysis:** The results derived from AC-ELISA and IHC using two enzyme labels (AP and P) were analyzed by Z-test using statistical software package STATA 9.1 (College state, Texas). P< 0.05 was considered significant.

**RESULTS**

Out of total tested samples (n=323) of old aged group B, 6.19% (n=20) samples showed < 1:2 SN antibody titers while 9.9 % samples (n=32) were found with SN antibodies titers ranging from 1:2 to 1:64. On the other hand, 271 samples (83.9%) were excluded from the study due to having high SN antibody titers (1:128 to 1:2048). Therefore, based on SN test, a total of 52 samples of group B and all of group A were further processed by AC-ELISA and IHC. Two ear notch biopsies originating from each of group A and B tested positive by AC-ELISA with S/P ratios of 0.704, 0.822, 0.921 and 0.395, respectively. On follow up testing, all the positive animals in each of the two groups remained positive confirming that these animals were truly PI animals, rather than acute transiently infected (Table1).

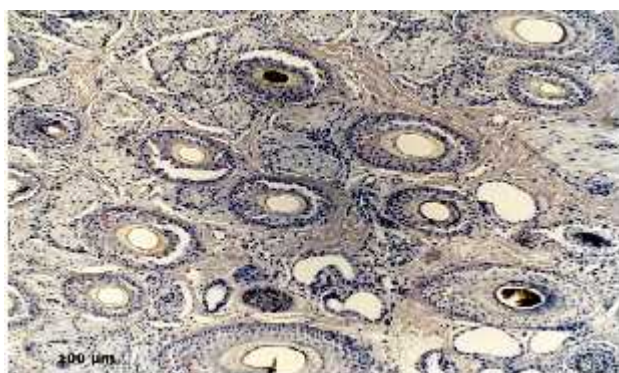
From tested ear notch biopsies (n=198), 2 EN biopsies, each of group A (1.37%) and B (3.84%) showed fine granular red precipitates in the follicular and hair

bulb cells with alkaline phosphates conjugate (AP) (Table 1, Fig 1A) confirming positivity. However Peroxidase (P) - detection system was found unable to differentiate between BVDV positive and negative skin biopsy sections as indistinguishable brown precipitates were observed in both known positive and negative tissue sections (Fig: 2A, 2B). No staining signals were observed in epidermal cells of positive skin biopsies as well as negative control slides (Fig: 1B, 1C, 1D). On follow up testing, all the 4 skin biopsies of positive animals remained positive confirming PI (Table1). To determine the genotype of prevailing BVDV, all the 4 positive skin biopsies were subjected to real time RT-PCR after testing probes specificity and sensitivity of PCR machine. 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 any cross reactivity. Only genotype 1 of BVDV was detected in positive EN biopsies.

**Table1. Comparative efficacy of AC-ELISA and IHC**

Tests	Positive samples			Positivity (%)			Agreement
	1 <sup>st</sup> Testing (N=198)		2 <sup>nd</sup> Testing (N=4)	1 <sup>st</sup> Testing		2 <sup>nd</sup> Testing	
	Group A (n=145)	Group B (n=52)	Group A +B (n=2+2)	Group A	Group B	Group A +B	
<b>ELISA</b>	2	2	4	1.37	3.84	100	100%
<b>IHC-AP</b>	2	2	4	1.37	3.84	100	100%
<b>IHC-P</b>	0	0	0	0	0	0	100%

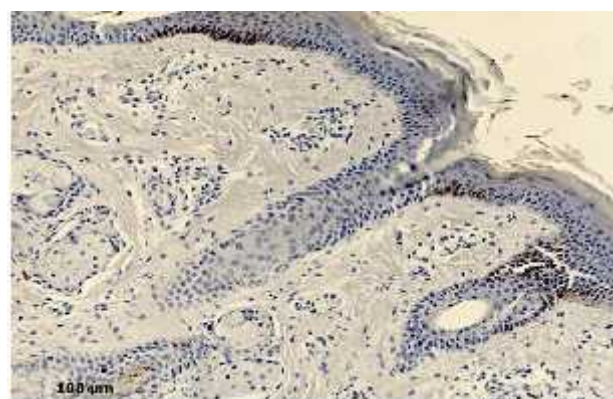
IHC-AP- immunohistochemistry with alkaline phosphatase label, IHC-P- immunohistochemistry with peroxidase label



**Fig 1. (A, B, C, D): Immunohistochemical staining of ear notch biopsies by Alkaline phosphatase-naphthol detection kit**

To validate the results of AC-ELISA and IHC for detecting all persistencies using EN biopsies, serum samples of both groups, when subjected to real time RT-PCR, yielded inconsistent results with those obtained by two assays on first round of EN testing. Five serum sample (3.42%) of group A and 2 (3.84%) of group B were tested positive on first round of testing. However, on follow up testing, two serum samples of group A turned negative depicting acute transient infection, while two samples of same animals which were tested positive

by AC-ELISA and IHC on EN, were consistently remained positive confirming presence of 2 PI animals. Data of one calf was excluded because of its death before second round of testing. This study concluded that for detection of BVDV persistencies, a complete harmony was observed among AC-ELISA, IHC and real time RT-PCR assays (data not shown). Furthermore, only genotype 1 of BVDV was found in the positive samples.



**Fig 1. (A): Skin biopsy of PI Animals . Immunohistochemical staining (red precipitates) of BVDV antigen within follicular, and hair bulb cells. 10x, Bar \_ 100 μm**

A non-significant difference ( $p > 0.05$ ) was observed between IHC-AP and AC-ELISA, for detection of BVDV from persistently infected animals, whereas there was a significant difference ( $P < 0.05$ ) between IHC-AP and IHC-P. On the basis of aforementioned findings, it could be concluded that both IHC-AP and AC-ELISA are equally suitable ( $P > 0.05$ ) while IHC-P is not ( $P < 0.05$ ).

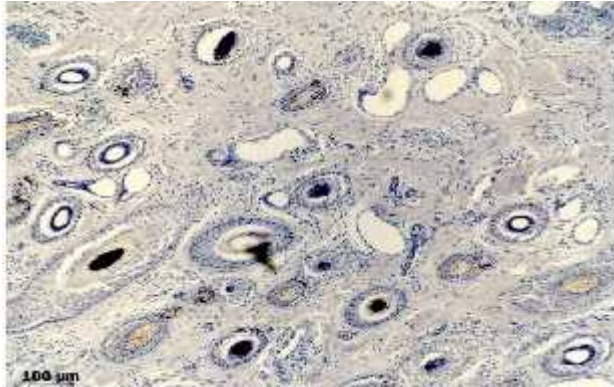


Fig1. (B): Skin biopsy of PI Animals. No immunohistochemical staining in the epidermal cells. 10x, Bar \_ 100 µm



Fig 1. (C): BVDV negative skin biopsy. No immunohistochemical staining in the follicular, and hair bulb and epidermal cells. 10x, Bar \_ 100 µm

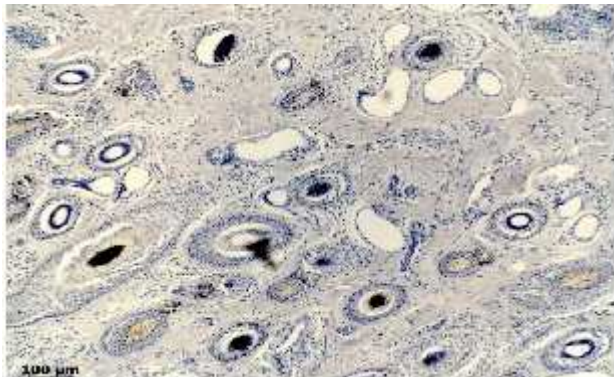


Fig 1. (D): BVDV negative skin biopsy. No immunohistochemical staining in the epidermal cells. 10x, Bar \_ 100 µm



Fig 2. (A, B): Immunohistochemical staining of ear notch biopsies by Peroxidase-DAB detection kit.

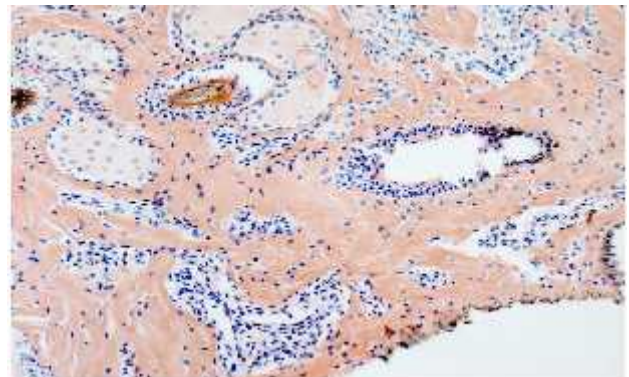


Fig 2. (A): Known positive BVDV infected skin biopsy. Immunohistochemical staining (brown precipitates within follicular and hair bulb cells. 20x, Bar \_ 100 µm

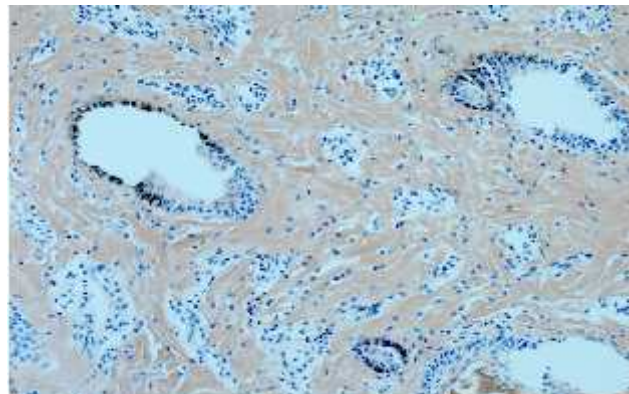


Fig 2. (B): Known BVDV negative skin biopsy. Immunohistochemical staining (brown precipitates within follicular and hair bulb cells. 20x, Bar \_ 100 µm

## DISCUSSION

The groups were formulated due to initial screening test by SNT, and the rationale for this was proven valid, since as expected the PI animals older than 6 months of age remained immunotolerant, and they did

not show specific anti-BVDV antibodies during their life span. Contrary to this, animals below 6 months of age may have had passive maternal antibodies in the course of persistency, if the mother had been transiently infected during later stages of pregnancy and passed the virus to the fetus after immuno-competency was established. While bovine fetus can form specific antibody as early as 120 days of gestation, competency of a bovine fetus to form specific BVDV antibody capable of curbing establishment of BVDV persistence was reported between 175 and 190 days of gestation (Chase *et al.*, 2007). Thus, these young animals were considered not be suitable for pre-screening by SNT serology. Because of this, only SNT prescreened samples of group B showing no or low titres of specific BVDV antibody, and all of group A, were tested for BVDV persistency.

In this study, AC-ELISA was found unable to detect transiently infected animals as a complete agreement between first and follow up testing was observed, confirming that these animals were truly PI animals, rather than acute transient infections (Table 1) and this was also proved by real time PCR on serum samples. Nevertheless on first round of testing, a significant discrepancy between AC-ELISA on ear notch biopsies and real time RT-PCR on serum samples collected from younger animals (group A) was observed. Three ear notch biopsies and 5 serum samples were found positive by AC-ELISA and PCR respectively but on follow up testing, a complete agreement between AC-ELISA on ear notch biopsies and PCR on serum samples has been observed. The two animals out of five, sera of which were found positive on first round of PCR testing and turned negative on follow up testing, were transiently infected animals. Bovine viral diarrhea virus status of one positive calf that died due to respiratory disease before collection of the second samples remained doubtful. All immunocompetent postnatally infected animals seroconvert and subsequently clear the virus for life; therefore, the only negative consequence of postnatal transient infection would be infection of susceptible pregnant cattle before 120 days of gestation (Potgieter, 1995). The discordance observed between serum and ear notch biopsies during this analysis, is in agreement with the previously reported findings by Hilbe *et al.*, (2007) who also identified transiently infected animals in three cases by Real Time RT-PCR in first round serum samples, but not in skin biopsies. Bovine viral diarrhea virus did not appear in the skin tissues when a low dose of virus is used to infect the animals (Ridpath *et al.*, 2002). Acutely infected animals do not have a significant amount of antigen in skin tissues in the vast majority of cases and this may be one of the possible reasons of not detecting transiently infected animals using ear notch biopsies by AC-ELISA (Saliki and Dubovi, 2004). However, Cornish *et al.* (2005), detected few acutely infected animals by using AC-ELISA on ear notch

samples. Our failure to detect any acutely infected by standing animals by AC-ELISA may be due to the presence of insufficient amount of viral antigen, below the sensitivity threshold of AC-ELISA.

In our study, though AC-ELISA on EN failed to detect acute transient animals but correctly detected all persistent animals, thus would have no negative implications on the outcome of the BVDV control effort through identification and culling of PI animals.

Suitability of immunohistochemistry for detection of BVDV persistent infection in melanin rich skin biopsy sections was investigated with the help of monoclonal antibody, and subsequently conjugates labeled with either peroxidase or alkaline phosphatase. These methods were compared for its reliability with that of antigen capture ELISA. The monoclonal antibodies used in this study were previously established to detect diverse BVDV isolates by reacting with the Erns protein, a highly conserved gene among pestiviruses (Donis *et al.*, 1988; Haines *et al.*, 1992; Silva-Krott *et al.*, 1994).

Alkaline phosphates conjugate (AP) system was found competent while Peroxidase (P)- detection system was proved incapable in our hands to differentiate between BVDV positive and negative skin biopsy sections, because of the mimicry of the reaction product by melanin. When evaluating the staining of these sections, it is important to realize that these sections are from tissues of PI animals and not from transiently infected or mucosal disease animals, as the pattern of staining in ear notches of those animals is different (data not shown). The epidermal cells of positive skin biopsies showed no immunohistochemical staining which is only partially in agreement with the findings of Cornish *et al.* (2005) and Luzzago *et al.* (2006), who reported specific staining signals in the follicular cells along with hair bulb and epidermal cells. This slight variation might be due to presence of different BVDV genotype, as in the study of Cornish *et al.* (2005) genotype 2 of BVDV was isolated in majority of the case, but in this study all the 4 positive samples were of genotype 1. As far as the study of Luzzago *et al.* (2006) is concerned, the genotype and subtype of BVDV was unknown and furthermore, he used a different kit in his study. We concluded from our work that AP label performed better than peroxidase on the melanin rich tissues, which is in agreement with Haines and Chelack, (1991). In our study no acutely infected animal was detected by IHC and this was also proved by real time RT-PCR on serum samples collected from same animals which is in line with the findings observed by various researchers (Ridpath *et al.*, 2002; Fulton *et al.*, 2006; Hilbe *et al.*, 2007). Contrary to this, Njaa *et al.* (2000) and Cornish *et al.* (2005) did report some acutely infected animals using ear notch biopsies. The discrepancy with Njaa *et al.*, 2000 might be explainable by the nature of the infection, as our animals were naturally infected while the animals in the above

study were experimentally infected, possibly with high amount of the challenge virus. Genotype variations may have been responsible for the findings of Cornish *et al.*, 2005. Furthermore, as Saliki and Dubovi 2004, proposed, acutely infected animals may contain lower amount of antigen in EN tissue, thus enabling interpretation of only one positive ear notch by immunohistochemistry, as indicative of PI.

Peroxidase conjugate yielded brown precipitates in all ear notch sections of our Holstein cattle, and these were indistinguishable from appearance of melanin deposits in the skin. Attempts were made to distinguish brown product from melanin by replacing the Hemotoxylin counter stain, with Giemsa stain, however the problem could not be solved to satisfactory level. This experience is in agreement with Haines and Chelack, 1991; Taylor *et al.*, 2002, but it is in disagreement with several researchers who have reported peroxidase based IHC method as a reliable technique for the detection of BVDV persistent infection (Groom and Keilen, 2002; Mahlum *et al.*, 2002; Hilbe *et al.*, 2007). The discrepancies are difficult to reconcile. While there may have been differences in the commercially procured detection kits, we speculate that the differences between our study and studies of those who found peroxidase IHC a suitable, may have been mainly due to differences in types of skin pigmentation between Holstein cattle in our study, and breeds employed in the studies in which peroxidase conjugate was found to yield useful results.

**Conclusions:** On the basis of aforementioned findings, it could be concluded that both IHC-AP and AC-ELISA are equally suitable ( $P>0.05$ ). However, IHC is labor intensive but AC-ELISA was proven to be quick, cheap, and easy to perform and interpret.

**Acknowledgements:** We would like to thank HEC, Pakistan for its generous support and funding to complete this research study under Ph.D. split program. Thanks are due to the University Management of Project entitled "Establishment of dairy and poultry animals training and research centers at Ravi Campus, UVAS, Pakistan". Furthermore, we appreciate the efforts of Dr. Toki Iwamoto and administration of AVC, UPEI, Canada for their invaluable support during execution of this research work.

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