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EVALUATION OF THE EFFECTS OF LONG-TERM STORAGE OF BOVINE EAR NOTCH SAMPLES ON THE ABILITY OF TWO DIAGNOSTIC ASSAYS TO IDENTIFY CALVES PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHOEA VIRUS

By

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A thesis submitted in partial fulfillment of the requirements for the Masters degree in Veterinary Tropical Diseases

In the

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July 2009

PREFACE

Declaration by Student

I Firdaus Khan declare that this dissertation is my own work carried out under the supervision of Professor M. Van Vuuren of the University of Pretoria and is in accordance with the requirements of the University for the award of the Master of Science degree in Veterinary Tropical Diseases.

…… ……
Date Signature

ACKNOWLEDGEMENTS

All praises and thanks are due to the Almighty. This dissertation would not have reached completion if it weren't for His will.

To my mother, thanks for all the prayers - words can't express how grateful I am to you. Thanks for being my pillar of strength. To my husband, thanks for all your patience and being my 'stress reliever', your words of comfort and encouragement always kept me motivated. To my entire family, thanks for believing in me.

To my supervisor, Professor Moritz van Vuuren, my sincere thanks for the quick responses to my emails, all your assistance and valuable input.

To my co-supervisor, Dr Johan Vorster at Vetdiagnostix- Veterinary Pathology Services – You have played a phenomenal role in conceiving this project. I wish to express my gratitude for your ongoing support and significant contributions and suggestions throughout this study. Thank you for this great opportunity at Vetdiagnostix.

Thanks to Dr Rick Last at Vetdiagnostix- Veterinary Pathology Services, for encouraging and affording me the opportunity to do my MSc, and for using Vetdiagnostix facilities to complete my research work.

Thanks to South African Veterinary Foundation for sponsoring the research project.

To all the staff at Vetdiagnostix, – Thanks to each of you for helping out whenever I needed assistance in the Serology section.

Thanks to Sparta Beef Farm and Karan Estate for donating samples.

To Dr Mapham, my sincere appreciation for your help with statistics and taking the time to review my work.

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ABSTRACT

Research aimed at optimizing diagnostic laboratory procedures is central to the development of effective bovine viral diarrhoea virus (BVDV) control programmes. BVDV is a single-stranded RNA virus that crosses the placenta to infect foetuses resulting in reproductive losses due to foetal death or persistently infected (PI) calves that usually die early in life. Persistently infected animals are widely accepted to be the primary reservoir of BVDV and the largest source of BVDV infection. Persistently infected animals that survive calfhood are at risk of developing mucosal disease in later life which is a severe and usually fatal condition. In addition, persistently infected calves that become replacement heifers in the herd may experience significant morphological changes that occur in the ovaries which can result in impaired reproductive performance. This poses important challenges to overall animal/herd health and causes losses to the cattle industry. Long-term storage of bovine ear notch samples from calves persistently infected (PI) with BVDV may affect the ability of diagnostic assays to efficiently detect the virus. This study assessed the effects of 1) long-term storage of formalin-fixed samples at room temperature to detect BVD viral antigen with the aid of immunohistochemistry (IHC), 2a) long-term storage of fresh ear notch samples kept at -20℃, and 2b) long term storage of phosphate-buffered saline (PBS) ear notch supernatant kept at -20℃ on the ability of an antigen-capture ELISA (AC-ELISA) to detect viral antigen. Previous studies have verified 100% sensitivity for both AC-ELISA on ear notch supernatant and immunohistochemical testing of ear notches to detect BVDV provided that samples are properly collected and stored. In this study, ear notch samples from seven animals were subjected to prompt formalin fixation and fresh samples to prolonged storage at -20℃. Frozen ear notches and ear notch supernatant yielded positive results on AC-ELISA for the duration of the study, i.e. 6 months, and OD values remained significantly within range. There was no significant difference between storing fresh ear notch samples and PBS ear-notch supernatant at -20℃. However, positive IHC staining on formalin-fixed ear notches started to fade away between day 17 and day 29 when stored at room temperature. We conclude that fresh ear notches could safely be stored at -20℃ for a period of 6 months for detecting BVD viral antigen at a later stage.

CHAPTER 1

1. INTRODUCTION

1.1 Motivation for the research project

Long-term storage of bovine ear notch samples from calves persistently infected (PI) with bovine viral diarrhoea virus (BVDV) may affect the ability of diagnostic assays to efficiently detect the virus. BVDV infection contributes to a variety of economically important disease syndromes in cattle. It has become a priority for veterinary and livestock associations to adopt measures to efficiently control BVDV. The accessibility to effective diagnostic methods is thus essential in this endeavour. Research aimed at optimizing diagnostic laboratory procedures is central to the development of effective control programmes. These findings will be useful to veterinary diagnostic laboratories and practitioners involved with design and implementation of BVDV biosecurity programmes in beef cattle farming.

1.2 Aims and objectives

Aims

- To determine the effects of storing ear notch samples in formalin for a prolonged period of time.
- To determine the effects of storing ear notch samples at -20℃.
- To determine the effects of storing PBS ear-notch supernatant kept at -20℃.

Objectives

- To study the effects of storing ear notch samples in formalin over a period of 6 months with the aid of immunohistochemical procedures to detect viral antigen.
- To study the effects of storing ear notch samples at -20℃ over a period of 6 months with the aid of an antigen capture-ELISA to detect viral antigen.
- To study the effects of storing PBS ear notch supernatant kept at -20℃ over a period of 6 months with the aid of an antigen capture-ELISA to detect viral antigen.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Background

Bovine viral diarrhoea (BVD) is an important viral disease and of major concern for cattle farmers and bovine practitioners as it causes livestock production losses worldwide. Persistently infected animals are widely accepted to be the primary reservoir of BVDV (Bhudevi & Weinstock 2003; Larson *et al*. 2005) and the largest source of BVDV infection (Njaa *et al*. 2000; Kennedy *et al*. 2006). Optimal testing procedures and thorough research has therefore become paramount to assessing and dealing with the economic losses associated with animals persistently infected with BVDV. The disease cannot be controlled with vaccinations alone and therefore continuous laboratory testing followed by removal of persistently infected animals will keep herds free of BVDV infections (Cornish *et al*. 2005; Larson *et al*. 2005).

BVDV is a single-stranded RNA virus that crosses the placenta efficiently to infect foetuses resulting in reproductive losses due to foetal death or calves that usually die early in life. Foetuses infected *in utero* between days 40 and 125 (Potgieter 2004; Larson *et al*. 2005; Kennedy *et al*. 2006) of gestation become immunotolerant to the virus and after birth are believed to shed the virus for life (Gripshover *et al*. 2007). These persistently infected calves carry very high viral loads, secreting high levels in all body secretions and excretions for a long period of time. Animals in contact with them become acutely infected and these infections are coupled with immunosuppression that gives way to opportunistic pathogens to further cause disease and reduce overall profits (Gripshover *et al*. 2007). There are two ways PI calves are produced: 1) susceptible pregnant cows infected with the virus, from any source, at 1-4 months of gestation, and 2) BVDV PI cows that become pregnant. Persistently infected animals that survive calfhood are at risk of developing mucosal disease in later life which is a severe and usually fatal condition. In addition, persistently infected calves that become replacement heifers in the herd may experience significant morphological changes that occur in the ovaries which can result in impaired reproductive performance (Potgieter 2004).

2.2 Diagnostic assays available for BVDV

Persistently infected animals represent a significant threat to the cattle industry and poses important challenges to overall animal/herd health. Individual animal testing is key in identifying and eliminating PI calves to prevent BVDV spread in a herd. There are numerous methods that have been developed to identify PI cattle such as virus isolation, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry (IHC) and several antigen- and antibody Elisa's (Kennedy *et al*. 2006; Hilbe *et al*. 2007). Virus isolation and RT-PCR are costly when used on individuals in a population, time consuming and can be technically demanding whereas IHC and antigen capture-ELISA (AC-ELISA) are cost effective methods. Immunohistochemistry takes 3-5 days to process whereas AC-ELISA uses less than 4 hours of laboratory time. Ear notches are easy and quick to sample. Maternal antibodies do not interfere with detection of the virus in ear notches and has thus become the sample of choice for testing (Gripshover *et al*. 2007; Kuhne *et al*. 2005). Previously documented studies have verified 100% sensitivity for both AC-ELISA on ear notch supernatant and immunohistochemical testing of ear notches to detect the presence of BVDV (Cornish *et al*. 2005; Kennedy *et al*. 2006) provided that samples are properly collected and stored (Larson *et al*. 2005). Kennedy *et al*. (2006) found that there was no difference in the ability of IHC and AC-ELISA to detect positive samples in individual samples tested. Thus, current diagnostic approaches in identifying PI calves predominantly makes use of ear notch testing with AC-ELISA and IHC. These assays depend on specific monoclonal antibodies which target the E^{rns} glycoprotein of BVDV (Gripshover *et al*. 2007).

Researchers have also established the use of RT-PCR pools followed by AC-ELISA and IHC on individual samples in the pool to efficiently screen herds and identify suspect animals (Kennedy *et al*. 2006). However, Ridpath *et al*. (2006) cautions against pooling of samples in surveillance programmes. Pooling of 10 samples, where a sample pool includes one positive and nine negative samples, resulted in the failure to detect 10% of the positive samples used in their study. Similarly, pooling of 100 samples, where the sample pool included one positive and 99 negative samples, resulted in failure to detect over 50% of the positive samples.

2.3 Critical review of published research

The effects of sample handling and storage on the ability of diagnostic assays to detect BVDV virus have not been clearly documented. Recently, researchers have begun exploring the effects of various storage and sampling methods that could ultimately influence the results obtained from diagnostic assays. Reed *et al.* (2008) found little effect of storing samples at room temperature for prolonged periods on the sensitivity of the AC-ELISA. All samples tested positive regardless of time stored at room temperature. However, Ridpath *et al*. (2006) and Ushijima & Cleveland (2005) reported reduced antigen detection on AC-ELISA at exposure to high temperatures and drying. Similarly, Miller *et al*. (2005) reported on reduced IHC antigen detection when samples were exposed to room temperature followed by formalin fixation. Exposure to room air for even 1 day resulted in desiccated samples that made histological processing difficult and produced false-negative IHC results. Prompt formalin fixation and histological processing is recommended by Miller *et al*. (2005). Their findings advocate storage in formalin for up to 1 month but caution against false-negative IHC reactions resulting from fixation longer than 36 days. A study that looked at detecting BVDV in formalin-fixed paraffin embedded tissue sections by RT-PCR showed decreases in signal strength when samples were fixed for more than 24-48h or when stored at room temperature following fixation after 74 h (Bhudevi & Weinstock 2003). These studies are few and lack uniformity in results. In our study we aim to determine the effect of storage of ear notch samples in formalin at room temperature on the ability of IHC to detect the virus over a period of 6 months.

Numerous studies have described the use of PBS when detecting BVDV in ear notch samples (Reed *et al*. 2008; Gripshover *et al,* 2007; Ridpath *et al.* 2006; Kennedy *et al*. 2005; Cornish *et al*. 2005; Larson et al. 2005; Ushijima & Cleveland, 2004; Brodersen *et al*. 2002) but no published research has indicated its effect on the accuracy of the AC-ELISA to detect the antigen. Ridpath *et al*. (2006) prepared ear notch extracts by soaking them in PBS for 60 min. They evaluated the effects of storing ear notch extracts and found no significant differences between storage at -20°C; -4°C; and 25°C for seven days on the ability of the AC-ELISA to detect antigen. Brodersen *et al*. (2002) demonstrated BVDV antigen in dilutions of extracts of fresh skin biopsies for an AC-ELISA. Samples were either frozen at –80°C or not frozen in either PBS or tissue diluent buffer. Mean corrected OD values differed significantly for each dilution and were greatest for tissues diluted 1:1. The

effect of using different tissues showed that the mean corrected OD from frozen samples (at 1:1 dilution) was significantly higher than non-frozen tissues. Kennedy *et al.* (2006) compared RT-PCR ear notch pools and individual AC-ELISA tests using the same PBS supernatant solutions for both tests. Both methods correlated 100% in detecting suspect PI animals. The purpose of our study was to document the use of prolonged storage of fresh ear notches and consider the effects of prolonged storage of PBS ear notch supernatant at -20°C on the ability of AC-ELISA to detect BVD viral antigen.

2.4 Null (H_O) and Alternative (H_A) Hypotheses

1a) H_O: Long-term storage of ear notch samples in formalin will not be associated with reduced IHC antigen detection on samples tested on the first day and last day of storage.

H_A: Long-term storage of ear notch samples in formalin will be associated with reduced IHC antigen detection on samples tested on the first day and last day of storage.

2a) H_O: Long-term storage of ear notches kept at –20°C will not be associated with reduced performance of the AC-ELISA to detect BVDV antigen on the first day and last day of storage.

H_A: Long-term storage of ear notches kept at –20°C will be associated with reduced performance of the AC-ELISA to detect BVDV antigen on the first day and last day of storage.

2b) H_O: Long-term storage of PBS ear notch supernatants kept at -20°C will not be associated with reduced performance of the AC-ELISA to detect BVDV antigen from the first and last day of storage.

H_A: Long-term storage of PBS ear notch supernatants kept at -20°C will be associated with reduced performance of the AC-ELISA to detect BVDV antigen from the first and last day of storage.

CHAPTER 3

3. METHODOLOGY

3.1 Sampling

An initial screening process, between 05 Aug – 08 Oct 2008, (Table 1) was conducted in the quest to identify PI animals from Sparta Beef Farm. However, animals died very soon after they tested positive. This made a second round of sampling, within at least 2 weeks, increasingly difficult. For purposes of this study, it was therefore decided to forgo the follow-up second testing and eliminate positive animals after a single initial positive test. Three animals from Karan Estate, previously identified with IHC as PI animals by a different laboratory, were donated to this study on the 18th of September 2008. This marked the beginning of the study. On the 15th of October 2008, four pairs of BVDV positive animal ears, previously tested with AC-ELISA, from Sparta Beef Farm were added to the study. These remained positive on both IHC and AC-ELISA on the first test of the study hence confirming their PI status. Ears were labelled with the corresponding calf numbers to assist with easy identification. In both cases, one ear from each animal was promptly placed in 10% neutral-buffered formalin, and the other fresh ear set aside in cooler boxes for AC-ELISA procedures at the laboratory the next day.

Since animals were sampled on two different dates, the study was divided into Group 1 – animals 3, 7 & 8; and Group 2: animals 10, 11, 12 & 13. Both groups of ears were subjected to the same test protocol but differed in test dates only. Tests were run twice a month with ~15 day intervals between tests. Groups 1 and 2 were eventually run on the same days starting from day 70 and day 43 respectively.

Table 1: Number of BVDV positive animals identified during the screening process.

Date Tested	No. of Ear Notches	No. Positive
05/08/2008	39	2
12/08/2008	32	1
21/08/2008	38	0
26/08/2008	47	4
02/09/2008	37	1
09/09/2008	40	4
16/09/2008	41	1

3.2 Experimental design

Table 2: An overview highlighting the experimental design of the study.

GROUPS 1 & 2	
One ear from each calf	
Promptly fixed in 10 % formalin, followed by storage at room temp. and testing with IHC every fortnight for 6 months.	12 notches frozen, followed by prolonged storage at –20^{o}C and testing with ELISA every fortnight for 6 months.
	12 notches suspended in fluid overnight, followed by prolonged freezing of supernatant at –20^{0}C and subsequent testing with ELISA every fortnight for 6 months.

3.2.1. Immunohistochemistry

Ears remained in formalin for ~24hrs before arriving at the laboratory. On arrival, rectangular ear notches ~1.0 X 1.0cm were obtained from the ventral margin of the pinna for each animal, using sterile surgical blades. Ear notches were embedded in paraffin wax (Tissue-Tek® VIP™) in 6-chambered cassettes, sectioned at 5µm and mounted on poly-L-lysine coated slides. Internal negative and positive controls were included with each cassette. Slides were sent to the Faculty of Veterinary Science, University of Pretoria, Onderstepoort for staining. Briefly, tissue sections were de-waxed and then blocked with 3 % hydrogen peroxide for 15 min, followed by incubation with a 15c5 BVDV monoclonal antibody at a working dilution of 1:500. Antibody was invented by and obtained from Professor Edward J. Dubovi, Animal Health Diagnostic Centre, Cornell University. A Nova Red counterstain was used to show BVDV antigen in the sections. Positive and negative control slides were also included with each stain. Control slides were prepared from known positive and negative samples collected by the University. Immunohistochemistry was repeated approximately twice a month for 6 months.

3.2.2. Antigen Capture (AC) -ELISA

Upon arrival at the laboratory, each ear was cut in two parts for both ELISA studies. These were further divided into 12 pieces for each animal, measuring ~2.0 X 2.0 mm (HerdChek BVDV Ag/Serum Plus, IDEXX Laboratories B.V) for 12 test runs over 6 months and stored either fresh or as PBS ear notch supernatant at -20℃.

Preparation of reagents and test protocol was followed as described by IDEXX HerdChek* BVDV Ag Test Kit/Serum Plus.

On arrival, a fresh ear notch from each animal was submerged in 150µl of IDEXX Ear Notch Soaking Buffer in individual dilution tubes and allowed to soak overnight (12-18 hrs) at 4℃. The remaining 11 fresh ear pieces were stored at -20℃.

All test reagents were brought to room temperature the next day and ear notches were removed from the buffer solution and discarded. Briefly, detection antibodies were added to all wells of a microtitre plate coated with E^{rns} monoclonal antibodies. Positive and negative controls were added in duplicate to appropriate wells, followed by fifty microlitres of the ear notch supernatant buffer to the remaining wells. The plate was left in the incubator at 37°C for 2hrs before washing and addition of conjugate and substrate. The optical density (OD) values were measured at 450nm and recorded as the start OD for each animal. Frozen ear notches were thereafter tested approximately twice a month for 6 months using the same procedure described above.

On arrival, twelve ear notches from each animal, measuring 2.0 X 2.0 mm, were submerged in individual aliquots of 100µl 0.1 M PBS (pH 7.4) (Cornish *et al*. 2005). Ear notches were left to soak overnight (12-18 hrs) at 4℃. Earpieces were removed the next day and the PBS supernatant aliquots were stored at - 20℃ for the duration of the study. On day 3, a frozen aliquot for each animal was removed from the freezer, thawed and tested with the AC-ELISA, as described above. This procedure was repeated approximately twice a month for 6 months.

3.3 Data Analysis

3.3.1. Immunohistochemistry

A specialist veterinary pathologist examined stained slides. Positive reactions were identified by evident Nova Red staining in the epidermis and hair follicles of the ear. Slides were graded based on the intensity of antigen staining using a 3-tier grading of 2 (strong, dense), 1 (diminished, scattered) and 0 (none) (Webster et al. 2009).

3.3.2. AC-ELISA

Results were calculated as described by IDEXX HerdChek* BVDV Ag Test Kit/Serum Plus insert. For the assay to be valid, the difference between the positive control mean (PCx) and the negative control mean (NCx) must be greater than or equal to 0.150 OD. Also, the NCx must be less than or equal to 0.250 OD.

Calculation of NCx

$$NCx = \frac{NC1\ A^{450} + NC2\ A^{450}}{2}$$

Calculation of PCx

$$PCx = \frac{PC1\ A^{450} + PC2\ A^{450}}{2}$$

Calculation for test sample:

$$S - N = \text{Sample } A^{450} - NCx\ A^{450}$$

Interpretation of results

1. Samples with S-N values less than or equal to 0.2 are classified as negative for BVDV antigen.
2. Samples with S-N values higher than 0.2 but less or equal to 0.3 are considered suspect.
3. Samples with S-N values higher than 0.3 are classified as positive for BVDV antigen.

S-N values were computed with the aid of Microsoft Excel software.

OD values for Group 1 and 2 were plotted separately against days to show the presence of BVDV antigen over 6 months. A one-tailed paired t-test was performed to determine if there was a significant difference between OD values recorded on the first and last day for both test treatments. $P \leq 0.05$ was considered to be statistically significant.

CHAPTER 4

4. RESULTS

4.1 Immunohistochemistry

Table 3: IHC staining intensity table of bovine ear notches over 6-months storage in formalin.

		Days in Storage												
		1	**17**	**35**	**53**	**70**	**84**	**102**	**117**	**132**	**147**	**162**	**177**	
CONTROLS	**Positive**	2	2	2	2	2	2	2	2	2	2	2	2	
	Negative	0	0	0	0	0	0	0	0	0	0	0	0	
GROUP 1	**3**	2	2	2	0	0	1	1	1	0	1	1	1	
	7	2	2	2	0	0	0	1	1	0	0	0	1	
	8	2	2	2	0	0	0	1	1	0	1	0	1	
			1	**15**	**29**	**43**	**57**	**75**	**90**	**105**	**120**	**135**	**150**	**165**
GROUP 2	**10**		2	2	0	0	0	1	1	0	0	1	0	0
	11		2	2	0	0	0	1	1	0	0	0	0	0
	12		2	2	0	0	0	1	1	0	0	0	0	0
	13		2	2	0	0	0	1	1	0	0	1	0	0

Positive BVD antigen staining was graded as follows: 2 – strong, dense, 1 – diminished, scattered, 0 – no staining. Shaded areas highlight days on which both groups were processed for IHC. Strong and dense staining patterns within the cytoplasm of keratinocytes (epidermal), sebaceous epithelial cells, follicular epithelial cells, mononuclear cells in the dermis and smooth muscle cells were observed between days 1 – 35 (Group 1) and days 1-15 (group 2). A clear loss of antigen staining was noticed on days 53 and 29, respectively. Markedly diminished and scattered antigen staining was spotted on both groups from days 84 and days 75 respectively. Staining was predominant in the follicular epithelial cells and virtually no staining was present in the epidermis. Other cells that revealed positive staining included dermal mononuclear cells, smooth muscle cells and cartilage cells.

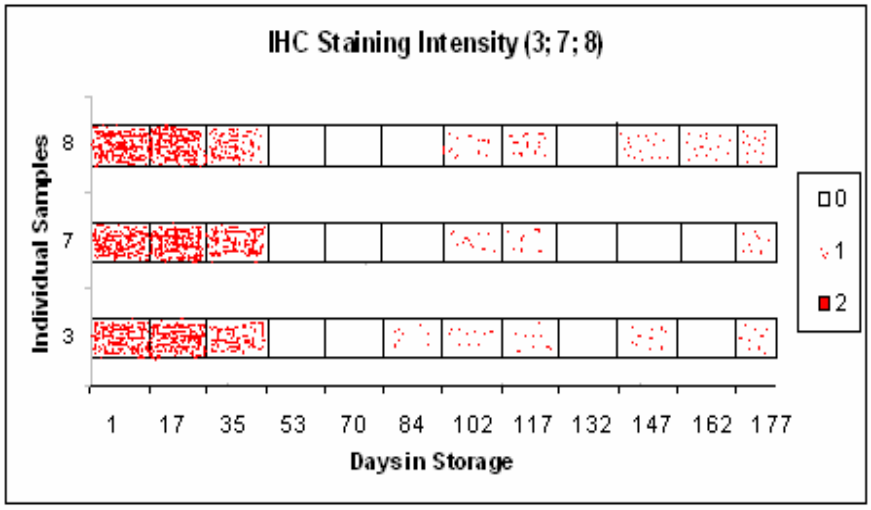

Figure 1.1: Graphical representation of tabulated data in table 4.1 for group 1 (3,7 & 8).

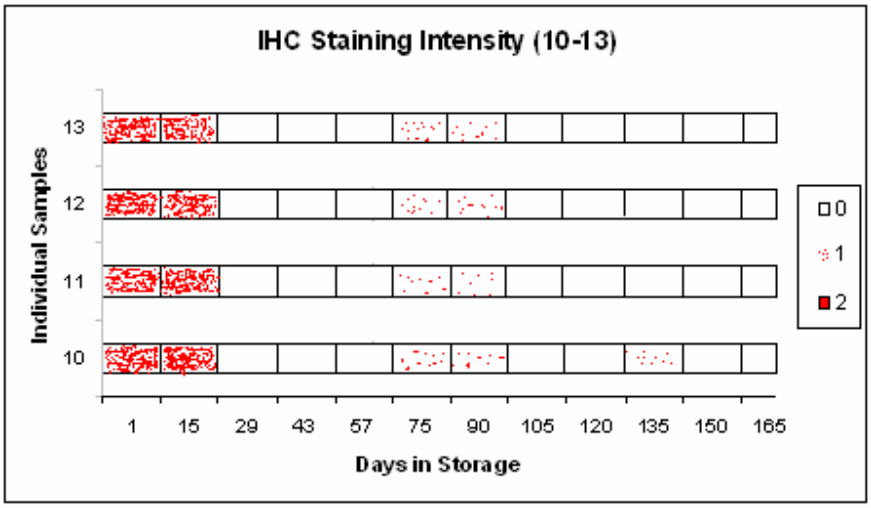

Figure 1.2: Graphical representation of tabulated data in table 4.1 for group 2 (10 -13).

The above two graphs display a graphical representation of strong; diminished; and loss of; antigen staining observed on individual ear notches over 6 months of storage in formalin.

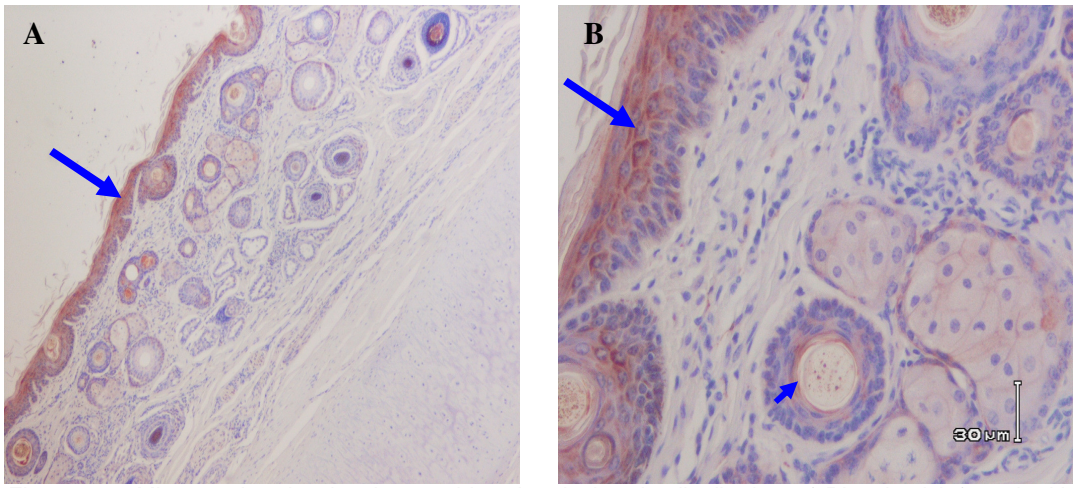

Figure 2.1: Micrographs showing immunohistochemical stain distribution of PI animal infected with BVDV. Staining is present in epidermis (arrows) and hair follicles (arrow head). Magnification: A = 40X; B = 200X.

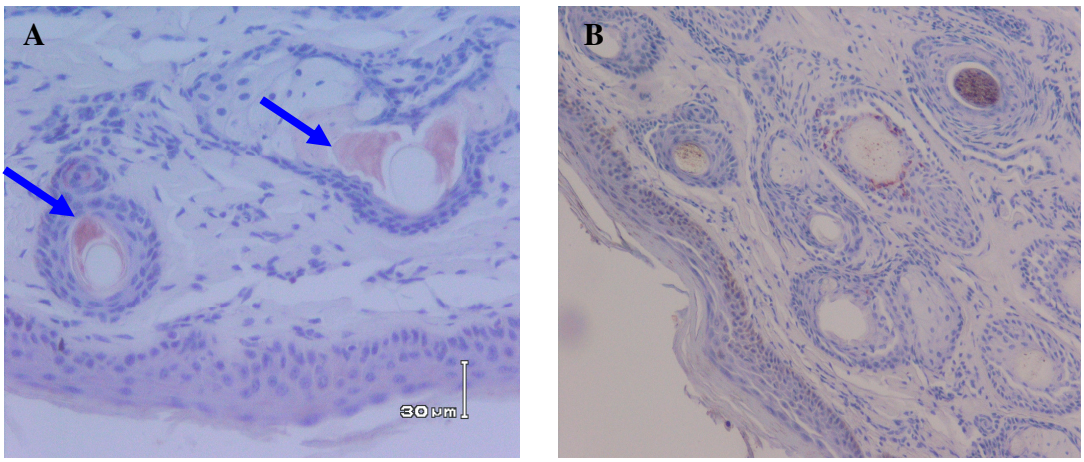

Figure 2.2: A- Micrographs showing non-specific staining (arrows). B- diminished staining (arrow head) on ear notches at day 84 (Group 1) and 75 (Group 2) in formalin. Magnification: A=200X; B=100X.

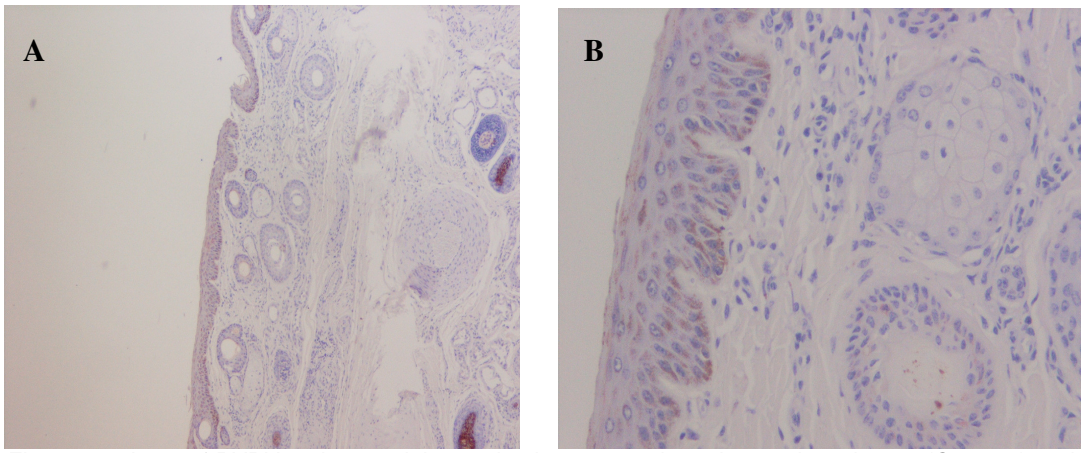

Figure 2.3: Loss of BVDV antigen staining on bovine ear notches observed on day 53 (Group 1) and day 29 (Group 2). Magnification: A = 40X; B = 200X.

4.2 AC-ELISA

Table 4.1: Corrected OD - S-N values obtained for ear notches stored at –20°C.

		DAYS IN STORAGE - EAR NOTCHES											
		0	***17***	***35***	***53***	***70***	***84***	***102***	***117***	***132***	***147***	***162***	***177***
GRP 1	***3***	4.08	3.99	4.21	3.67	3.45	3.86	3.95	3.63	3.23	3.62	3.62	4.07
	7	4.87	3.72	3.84	3.56	3.89	3.54	3.91	3.66	3.31	3.52	3.91	3.78
	8	2.87	2.36	3.47	2.58	2.74	2.32	2.95	1.82	1.71	1.83	2.64	2.27
		0	***15***	***29***	***43***	***57***	***75***	***90***	***105***	***120***	***135***	***150***	***165***
GRP 2	***10***	3.45	4.14	4.88	3.7	3.88	4.21	3.78	3.78	0.96	3.86	5.07	3.6
	11	3.7	3.5	3.81	3.49	3.68	4.76	3.69	3.91	1.03	3.76	3.8	3.52
	12	3.39	3.95	3.79	3.55	4.46	5.94	3.66	4.08	4.09	3.76	3.79	3.76
	13	3.65	3.91	4.14	3.31	4.18	4.64	3.79	3.57	3.71	3.85	3.8	3.61

A one-tail paired t-test was performed on corrected OD values obtained at the start (Day 0) of the study and the last day of storage.

Table 4.2: Corrected OD - S-N values obtained for ear notch PBS supernatant stored at –20°C.

		DAYS IN STORAGE - EAR NOTCH PBS SUPERNATANT											
		1	***17***	***35***	***53***	***70***	***84***	***102***	***117***	***132***	***147***	***162***	***177***
GRP 1	***3***	3.96	3.85	4.48	4.62	3.52	3.77	4.37	3.43	3.65	3.55	3.26	3.61
	7	1.76	2.82	2.77	2.87	2.76	2.86	2.79	2.58	2.05	1.89	2.31	1.83
	8	2.05	2.12	2.17	2	2.5	2.37	1.94	1.26	1.49	1.43	1.51	1.68
		1	***15***	***29***	***43***	***57***	***75***	***90***	***105***	***120***	***135***	***150***	***165***
GRP 2	***10***	3.87	4.17	4.47	4.04	4.18	3.66	3.62	3.88	3.47	3.71	4.05	3.74
	11	3.87	3.7	3.49	3.64	3.9	3.54	3.39	3.14	3.34	3.51	3.56	3.78
	12	3.72	4.85	3.58	3.85	3.99	3.78	3.6	3.86	3.49	3.62	3.91	4.01
	13	3.75	3.7	4.19	3.93	4.2	3.71	3.74	3.74	3.59	3.62	3.65	2.9

A one-tail paired t-test was performed on corrected OD values obtained on the first and last day of storage.

Table 4.3: T-test results for significant differences between positive OD values obtained at the start and last day of storage.

	KIT AC-ELISA	PBS AC-ELISA
GROUP 1	0.106	0.226
GROUP 2	0.287	0.24

There were no significant differences between OD values for all positive BVD ear notches tested on the first day samples arrived and the last day of storage. Samples remained positive throughout the storage period regardless of buffer treatments applied.

CHAPTER 5

5. DISCUSSION, CONCLUSION & RECOMMENDATIONS

5.1 Immunohistochemistry

Immunohistochemistry is a useful diagnostic tool for antigen detection and has been applied to detect various pathogens. While formalin provides excellent preservation of tissue architecture, formalin fixation is known to be a limiting factor for IHC. High concentrations of proteins and other solutes within tissue leads to the formation of a dense irregular network of cross-links and overfixation can render some epitopes inaccessible to immunoreagents (Webster *et al*. 2009), decreasing the sensitivity of an IHC test.

This study has shown that long-term storage of bovine ear notches in formalin is associated with reduced reactivity of BVD viral antigen with immunohistochemical staining procedures and therefore rejects the null hypothesis. The pattern of IHC staining for all animals on the first day of storage in formalin was strong and dense in epidermal and hair follicle epithelial cells, (Fig 2.1). In group 1, the distribution and intensity of staining decreased by the third test (Fig 1.1) and was completely lost by the fourth test (Fig 2.3). In group 2, positive staining ear notches were completely lost by the third test. Miller *et al*. (2005) advocated storage in formalin for up to 1 month but have cautioned against false-negative IHC reactions resulting from fixation longer than 36 days. Although an exact day cannot be established here, it is evident from both groups that BVDV antigen staining starts to fade away anytime between day 17 and day 29 when stored in formalin (Table 2). Figures 1.1 & 1.2 give a graphical representation of antigen staining on each ear notch as observed throughout formalin storage. Webster *et al*. (2009) evaluated the effect of prolonged formalin fixation on IHC detection of 61 different antigens. Their results suggest that long-term fixation has little effect on antigen detection ‘for most commonly used antibodies’ while other antibodies show ‘moderate to marked decreases in their detection abilities’ following long-term fixation. They found that moderate variations in fixation times up to 7wks should not significantly affect IHC results. Therefore it was concluded that it is likely that the effects of long-term formalin fixation would depend on the antibody, the antigen of interest and the antigen detection method.

The diminished and scattered appearance of antigen staining from day 84 (Group 1) and day 75 (group 2) (Table 2) could be attributed to one or a combination of the following factors discussed. Tissue and slide processing procedures could be responsible for the late appearance of positive antigen staining. False positive results could also be due to non-immunological binding of proteins or substrate reaction products. They may also be the result of endogenous biotin activity, a known source of non-specific staining in IHC procedures based on the biotin-avidin system (Mount & Cooper 2001). Biotin-avidin interactions form the basis for the identification of antigens in IHC. Also, autolysis will continue until all tissues are penetrated by formalin. Therefore, Webster *et al*. (2009) attributes the variations in immunoreactivity to the differing degrees of autolysis that usually occurs before fixation. Necrotic or degenerated cells can also exhibit non-specific staining. Non-specific staining is typically of a diffuse appearance. The reason for this discrepancy is uncertain considering that negative controls were approved. Ear notches were taken from different areas of a whole ear on each day of IHC processing and it is possible that variations in antigen concentration and distribution, and variation in tissues evaluated could have resulted in different protein-protein cross-links and differential effects of prolonged fixation (Webster *et al*. 2009). Similarly, weak follicular staining and no epidermal IHC reactions were also described by Miller *et al.* (2005) after 176 days in formalin.

Formalin fixation times are rarely standardized (Webster *et al*. 2009) and this variability lends itself to vast inconsistencies when interpreting IHC results, especially when results are unexpected. The subjective nature in interpreting immunohistochemical results is also a cause of inconsistency and open to interpreters' biases. The observations in this study reiterates the importance of proper optimization and standardization of antigen retrieval protocols for antibody and tissues used in IHC techniques (Webster *et al*. 2009) to obtain reproducible results every time.

From this study and a previous report (Miller *et al*. 2005) on prolonged formalin fixation on IHC detection of BVDV, the most favourable procedure to follow would be to

- promptly fix samples for no less than 24 hrs to minimize the risk of desiccated samples, and to minimize the risk of under fixation
- store in formalin for no more than 17 days to minimize risk of false negative antigen staining.

5.2 AC-ELISA

Skin biopsies have been shown to be useful for the diagnosis of BVDV and its use is widely accepted by researchers worldwide (Njaa *et al*. 2000; Cornish *et al*. 2005). Long-term storage of bovine ear notches kept at –20°C proved to be an efficient storage method for subsequent BVDV antigen detection. All samples remained positive over the 6-month storage period (Table 3.1) with no significant differences between start OD values and OD values obtained on the last day of storage (Table 3.3) therefore the null hypothesis of no difference is accepted. A similar finding of strong positive results and low variation in testing multiple samples from the same ear of a PI animal was reported by Kuhne *et al*. (2005). However, analysing OD values obtained on different days can lead to inconsistencies due its erratic nature affected by uncontrolled parameters in the laboratory. Corrected OD values, S-N values, confirmed positive results (Table 3.1 & 3.2).

The use of PBS as ear notch supernatant as an alternative to the manufacturer's soaking buffer, proved to be relatively reliable. Similar to frozen ear notches, all PBS ear notch supernatants remained positive over the 6-month storage period (Table 3.2) with no significant differences between OD values from the first day and last day of storage (Table 3.3) therefore the null hypothesis of no difference is accepted. It is common practice to maintain ear notches at –20°C (Gripshover *et al*. 2007; Larson *et al*. 2005) or PBS (Gripshover *et al*. 2007; Ridpath *et al*. 2006) during transport to a laboratory to prevent desiccation (Ushijima &Cleveland, 2005). The findings in this study contrast with a previous report of significantly higher mean OD values for ear notches frozen in PBS or tissue diluent buffer (Brodersen *et al.* 2002). In this study, start OD values for 4 (animals 3,7,8, & 11) out of 7 unfrozen ear notches (Table 3.1) were higher than ODs obtained after ~ 15 days storage at -20 °C. Furthermore, OD values for frozen PBS ear notch supernatant in group 1 (Table 3.2) were lower than the ODs obtained from unfrozen kit buffer ear notch supernatants. (Table 3.1). Similarly, Ridpath *et al.* (2009) observed the lowest sample to positive (S/P) ratio in foetal ear punch samples stored at –20°C and the highest S/P values for samples stored at 37°C. It is not clear from the previous report and others (Reed *et al*. 2008; Gripshover *et al*, 2007) if samples are frozen with or without ear notches. The protocol followed here instructs overnight (12-18 hrs) soaking and conducting the test with only the supernatant. The effect of excessive soaking has not been documented and could be an element to be seriously considered in future studies.

Also, samples were frozen at –80°C (Brodersen *et al*. 2002) compared to –20°C in this study. Ridpath *et al* (2009) explains the possibility of the release of E^{rns} viral proteins during desiccation hence higher S/P values. The destructive effects of freezing (-80°C) PBS with the ear notch may also contribute to release of viral proteins after cell lysis.

The ultimate goal of fixation is to preserve tissues and antigenicity. While formalin fixation allows that, long-term storage in formalin is comparatively limited precluding any chance of further investigation of the sample at hand. The risk of misinterpreting a true positive PI animal is more than likely, even at two weeks in formalin. It is therefore recommended that ear notches be frozen at –20°C for future BVDV investigations. Since kit reagents are limited and sufficient for just the plates provided, it is suitable to use a 0.1M phosphate buffered saline (pH 7.4) solution to obtain ear notch supernatant. This supernatant can be frozen at –20°C and later used for retrospective studies. The option of freezing ear notch supernatant may prove more reliable than freezing ear notch tissue which may affect the ability of the ELISA to detect the BVDV virus over time. Ozkul *et al*. (2001) compared 4 diagnostic tests for detecting BVD in buffy coat samples after long term-storage. Their findings indicate that RT-PCR is more suitable for detecting BVDV in frozen buffy coats stored over a year. This lower immunodetection sensitivity of the virus on other tests was attributed to the presence of toxic elements after autolysis following freezing and thawing.

The development of the ear notch AC-ELISA has paved the way for quicker, easier and reliable results with only 1 technician compared to IHC which demands time and a team to deliver. Unfortunately this study was only performed on 7 animals and further divided into 2 time frames; therefore results could not be interpreted collectively. In addition, the study was only conducted over 6 months and a longer storage period would be worthy of note for future examination. Furthermore, although antigen was successfully detected in all samples in frozen PBS ear notch supernatant throughout the 6-months, a longer storage period may also allow for detecting any differences between the buffers. This principle comes from the findings of Brodersen *et al*. (2002) where OD values differed significantly by each dilution and were greatest at 1:1. Since, IDEXX tissue diluent buffer concentration and constituents is unknown and is only speculated to be PBS, it could be possible that PBS varies considerably to the manufacturer's reagents. Consistent results from multiple studies are still required to reach firm conclusions.

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APPENDIX A

This appendix presents the raw OD values of the seven samples as obtained at 450nm.

Table 5.1: Raw OD value obtained for ear notches stored at –20°C.

		DAYS IN STORAGE - EAR NOTCHES											
		0	***17***	***35***	***53***	***70***	***84***	***102***	***117***	***132***	***147***	***162***	***177***
GRP 1	***3***	4.19	4.1	4.32	3.77	3.69	3.95	4.03	3.73	3.33	3.71	3.72	4.18
	7	4.98	3.83	3.95	3.67	4.14	3.63	4	3.76	3.41	3.61	4.01	3.89
	8	2.98	2.47	3.58	2.68	2.98	2.41	3.03	1.93	1.82	1.92	2.75	2.68
		0	***15***	***29***	***43***	***57***	***75***	***90***	***105***	***120***	***135***	***150***	***165***
GRP 2	***10***	3.55	4.28	4.99	3.95	3.97	4.29	3.88	3.89	1.05	3.96	5.18	3.72
	11	3.8	3.64	3.92	3.73	3.77	4.85	3.8	4.02	1.12	3.86	3.9	3.64
	12	3.48	4.08	3.9	3.79	4.55	6.02	3.77	4.19	4.18	3.86	3.9	3.87
	13	3.74	4.05	4.14	3.56	3.55	4.72	3.89	3.68	3.8	3.95	3.91	3.73

Table 5.2: Raw OD values obtained for ear notch PBS supernatant stored at –20°C.

		DAYS IN STORAGE - PBS EAR NOTCH SUPERNATANT											
		1	***17***	***35***	***53***	***70***	***84***	***102***	***117***	***132***	***147***	***162***	***177***
GRP 1	***3***	4.07	3.96	4.59	4.72	3.77	3.86	4.45	3.53	3.76	3.65	3.36	3.72
	7	1.87	2.93	2.88	2.98	3.01	2.95	2.88	2.68	2.16	1.98	2.41	1.94
	8	2.16	2.23	2.28	2.11	2.75	2.46	2.03	1.36	1.59	1.52	1.61	1.79
		1	***15***	***29***	***43***	***57***	***75***	***90***	***105***	***120***	***135***	***150***	***165***
GRP 2	***10***	3.97	4.31	4.58	4.29	4.26	3.74	3.73	3.99	3.56	3.8	4.16	3.86
	11	3.96	3.84	3.6	3.89	3.99	3.62	3.49	3.25	3.43	3.61	3.67	3.89
	12	3.82	4.98	3.69	4.1	4.08	3.86	3.7	3.97	3.58	3.72	4.02	4.12
	13	3.85	3.84	4.3	4.18	4.29	3.8	3.85	3.85	3.68	3.73	3.76	3.008