

**THE DIAGNOSIS AND PREVALENCE OF PERSISTENT
INFECTION WITH BOVINE VIRAL DIARRHOEA VIRUS IN
FEEDLOT CATTLE**

by

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DECLARATION

I, Thelma Meiring, hereby declare that the dissertation submitted herewith for the degree MMedVet (Pathology) at the University of Pretoria contains my own independent work and has not been submitted for any degree at any other institution.

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SUMMARY

Bovine viral diarrhoea virus infection is an important viral infection affecting the cattle industry today. The prevalence of this infection in South African feedlots is unknown.

Ear notch biopsies were collected from animals entering feedlots which appeared unthrifty, chronic poor doers, and animals entering the hospital pen with respiratory disease for the first time. One thousand and seventy four (1074) samples were collected from the first two categories and 616 samples from animals entering the hospital pen. Samples were processed with routine immunoperoxidase protocol. Serum samples were also collected when possible.

The first aim of this study was to determine the prevalence with the use of immunoperoxidase staining on ear notch biopsies. Overall 49 animals tested positive, 43 from the 1074 group and 6 from the 616 group. The prevalence of persistently infected cattle entering the feedlots was determined as 2.9%, which is higher than the rule of thumb that 0.5% of infected animals enter feedlots. Four percent were positive in the group of 1074 animals and one percent in those entering the hospital pen for the first time.

It was proposed by the author that persistently infected animals are at a greater risk to develop respiratory disease in the feedlot, but this was not supported by the data collected. There was thus no clear increase in respiratory disease in persistently infected animals.

The reliability of the immunoperoxidase stain as a diagnostic method to identify persistently infected animals was also evaluated. This diagnostic method proved to be reliable, but the pathologist needs to be aware of non-specific staining. During the course of the research it became apparent that in some cases mast cells in the dermis stain positive with both DAB and NovaRED stains. Positive staining in keratinocytes and hair follicle epithelium was not present and these cases were proven as negative for persistent infection. The specific cause of positive staining of mast cell granules remains unclear.

Only ten positive cases had serum samples on which ELISA tests for antigen and antibody were performed. All tests correlated well with the immunoperoxidase method except in four cases, where the animals were incorrectly diagnosed as positive due to the non-specific staining as described above. Immunoperoxidase staining on ear notch biopsies is thus a reliable diagnostic method to identify persistently infected animals with BVDV, but the pathologist must be aware of non-specific positive staining.

OPSOMMING

Bees virus diarree infeksie is 'n belangrike virale infeksie wat die bees industrie van vandag beïnvloed. Die prevalensie van die infeksie in Suid Afrikaanse voerkrale is onbekend.

Oorknip biopsies is geneem van verdagte diere met aankoms by die voerkraal, chroniese swak beeste en diere wat vir die eerste keer in die hospitaal kraal weens respiratoriese siekte opgeneem is. Een duised vier en sewentig (1074) monsters is van die eerste twee kategoriee geneem en 616 monsters van diere wat in die hospitaal kraal opgeneem is. Monsters is op roetiene wyse vir immunoperoksidase kleuring geprosesseer. Serum monsters is waar moontlik ook versamel.

Die eerste doel van die studie was om die prevalensie van permanente besmette draers te bepaal met behulp van immunoperoksidase kleuring op oorknip biopsies. Nege-en-veertig diere in totaal het positief getoets, 43 vanuit die eerste groep en 6 vanuit die tweede groep. Die prevalensie van permanente besmette draers wat in voerkrale opgeneem word is was 2.9% wat hoër is as die verwagte 0.5% wat deur die literatuur aangedui word. Vier persent was positief in die 1074 groep en 1% in die groep wat vir die eerste keer in die hospitaal kraal opgeneem is.

Dit is deur die navorser voorgestel dat permanente besmette draers 'n groter risiko het om met respiratoriese siektes in die hospitaal kraal opgeneem te

word, maar dit is nie deur die data bevestig nie. Daar was dus geen verhoging in die teenwoordigheid van respiratoriese siektes in geaffekteerde diere nie.

Die betroubaarheid van immunoperoksidase kleuring om permanente besmette diere met BVD te identifiseer is ook geëvalueer. Die metode is betroubaar gevind, maar die patoloog moet bewus wees van nie-spesifieke kleuring. Gedurende die navorsing het dit aan die lig gekom dat mastelle in die dermis positief kleur met DAB en NovaRED kleuring. Positiewe kleuring was nie in die epidermis of haarfollikel epiteel teenwoordig nie en die gevalle was negatief vir permanente besmetting. Die spesifieke rede vir positiewe kleuring in mastelle is steeds onduidelik.

Slegs 10 positiewe gevalle het serum monsters gehad vir ELISA teenliggaam en antigeen toetse. Die resultate het goed gekorrelleer met die immunoperoksidase kleuring, behalwe in 4 gevalle waar gevalle verkeerd as positief gediagnoseer is as gevolg van nie-spesifieke positiewe kleuring soos beskryf. Immunoperoksidase kleuring is dus 'n sensitiewe metode om permanente besmette draers met BVDV te identifiseer, mits die patoloog bewus is van nie-spesifieke kleuring wat mag voorkom.

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CHAPTER ONE

LITERATURE REVIEW

1. INTRODUCTION

1.1 The virus and effects

Bovine viral diarrhoea virus (BVDV) is a virus of the family Flaviviridae and a member of the genus *Pestivirus*. There are two biotypes, designated as non-cytopathic (NCP) and cytopathic (CP), depending on their effect on tissue culture cells. The NCP type is the most common and the most important and is also the one that is found in persistently infected (PI) animals. There is considerable antigenic diversity and cross-reactivity among isolates of BVDV. At least two genotypes exist, which are unrelated to biotype. Bovine viral diarrhoea virus has been divided into genotypes I and II, depending on antigenic and genetic differences (Kampa *et al.*, 2007). Non-cytopathic and cytopathic isolates exist within each genotype. Genotype I has recently been subdivided into two sub-genotypes BVDV1a and BVDV1b (Radostits *et al.*, 2000; Potgieter, 2004). Additional sub-genotypes have been proposed for strains that are unique to Southern Africa (Potgieter, 2004).

Bovine viral diarrhoea virus is one of the most economically important pathogens in the cattle industry today (Radostis *et al.*, 2000; Firat *et al.*, 2002; Van Vuuren, 2005; Houe *et al.*, 2006; Hilbe *et al.*, 2007). It causes a multitude of different diseases including the following:

1. A subclinical, benign diarrhoea (bovine virus diarrhoea).
2. Peracute highly fatal diarrhoea.
3. A haemorrhagic and thrombocytopenic disease.
4. Reproductive failure.
5. Fatal mucosal disease of PI animals, which are infected early *in utero*.
6. Abortions and malformations (Radostits *et al.*, 2000; Firat *et al.*, 2002).

The NCP type of BVDV virus is the predominant one in nature. Radostits *et al.* (2000) state that it is the only one that crosses the placenta to infect the foetus (Firat *et al.*, 2002). When the NCP virus infects the foetus between day 42 and 125, the immune system develops a tolerance towards the virus and does not develop an immune response (McClurkin *et al.*, 1984; Luzzago *et al.*, 2006; Hilbe *et al.*, 2007; Kampa *et al.*, 2007). The calves are born persistently viraemic and continue to shed virus for the rest of their lives. The neonatal mortality of PI calves is high, and they are often born weak (Moczygamba, 2003). Others are normal and healthy at birth. Fatal mucosal disease may develop between 6 and 24 months of age if the animal is superinfected with a homologous strain of a CP virus or if the NCP virus in the body mutates to a homologous CP virus (Radostits *et al.*, 2000; Potgieter, 2004).

The economic importance of BVDV results from abortion, congenital defects, still births, increased neonatal mortality, prenatal and postnatal growth retardation, sub-optimal reproductive performance, death from mucosal disease and premature disposal of PI animals (Radostits *et al.*, 2000; Firat *et al.*, 2002; Cornish *et al.*, 2005; Hilbe *et al.*, 2007). Economic impact specifically associated with feedlots includes expenses incurred for treatment, additional labour (care and movement of animals),

premature culling because of chronic disease (repeat treatments) and profit reduction due to reduced growth and performance during and after illness (Hessman *et al.*, 2009). First treatment for respiratory disease (as a result of stress and immune suppression caused by BVDV) costs approximately R80.00 per animal plus an additional standing time (time animals spend in feedlots prior to slaughter) of 21 days at a further cost of about R180.00. After the second treatment, standing time increases to 40 days and after the third to 70 days (Personal communication Dr. S Morris, 2005). If one estimates that in a high-risk group (a pen that has one PI animal) 30% of all animals may require treatment for respiratory diseases and additional standing time, veterinary medical costs and extra expenses incurred are significant. Not all animals recover, and, if treatment is discontinued after two or three treatments, animals might be euthanased or have to be sold at a loss. Mortality can vary from 1 to 5% in affected animals. Animals that require treatment for respiratory diseases gain 0.14-0.23kg per day less than healthy animals (Moczygamba, 2003). Thomson (Ishmael, 2003) stated that PI calves represented roughly 5.8% of all calves treated for respiratory diseases and accounted for 25% of the mortalities. Pens containing a single PI animal experience a pull rate (animals removed to hospital pen because of apparent illness) 33% higher than pens without PI animals. Pens adjacent to those with PI animals also experience a similar increase in morbidity rate/pull rate. Loneragan *et al.* (2005) found an even higher pull rate incidence of 43%. The prevalence of PI animals in an infected herd is normally 1-2%, and the incidence of mucosal disease amongst PI animals is 5%, but can be as high as 22% (Radostits *et al.*, 2000). The rule of thumb in the feedlot industry is that 1% of calves born per year in an infected herd are PI animals. About half of

these will die before weaning, which makes the industry's average intake rate of PI animals 0.5% (Ishmael, 2003).

Feedlots are particularly at risk of receiving PI animals. Persistently infected calves can present as stunted animals with an unthrifty coat, but not all PI animals are in poor condition and it is not possible to tell from the physical appearance of an animal if it is PI (Potgieter, 2004). The real dangers of PI animals lies in the fact that they are persistently viraemic, immunosuppressed and constantly/intermittently shedding virus, and are the main source of infection for other animals (Cornish *et al.*, 2005; Loneragan *et al.*, 2005; Van Vuuren, 2005; Luzzago *et al.*, 2006; Kampa *et al.*, 2007). Brock *et al.* (1998) and Moczygemba (2003) suggest that the levels of viraemia in PI calves are cyclic, and that higher levels of virus and shedding may occur under stressful situations, for example transport and entry into a feedlot. Niskanen *et al.* (2002) infected 10 calves with BVDV and bovine coronavirus (BCV) and put non-infected calves with them. None of the latter calves contracted BVDV, but all became BCV positive, suggesting that acutely infected animals do not secrete BVDV effectively and appear not to be a major source of infection for other animals, and that PI animals are the major source of BVDV infection (Potgieter, 2004).

Bovine viral diarrhoea virus infects the lymphoid system and is immunosuppressive (Ridpath, 2002; Potgieter, 2004) as it infects the dendritic or stromal macrophages that support and nurse lymphoid cells during their development. Damage to these cells destroys the lymphoid population, which leads to lymphopaenia and immunosuppression (Wren, 2001).

1.2 Diagnostic methods

A reliable, fast, diagnostic tool to identify the presence of BVDV antigen is immunoperoxidase staining of skin biopsies (Thur *et al.*, 1996; Houe *et al.*, 2006; Luzzago *et al.*, 2006; Hilbe *et al.*, 2007). Various researchers sampled skin from various locations on PI animals and found that results were identical irrespective of the location from which the sample was collected (Thur *et al.*, 1996). The presence of BVDV antigen in keratinocytes in the stratum basale and stratum spinosum has been documented in clinically normal PI cattle (DuBois *et al.*, 2000; Thur *et al.*, 1996; Bielefeldt Ohman, 1988; Sandvik, 1999). According to Njaa *et al.* (2000), BVDV has a tropism for lymphocytes, mononuclear phagocytes and epithelial cells. In their study of formalin-fixed skin biopsies, positive immunoperoxidase staining for BVDV was found in the cytoplasm of keratinocytes, sebaceous epithelial cells, mononuclear cells in the dermis and vascular smooth muscle cells. The epidermal staining was predominantly in the stratum basale and stratum spinosum and it occurred diffusely. The most prominent staining was found in the isthmus and infundibulum of hair follicles. Previous studies showed immunohistochemical (IHC) testing to be as reliable as virus isolation (VI) to detect PI cattle (Thur *et al.*, 1996; Broderson *et al.*, 1998). The advantages of the IHC staining are that tissues are fixed in formalin, and it is more rapid and economical than virus isolation.

There is little chance of identifying an acutely infected animal as falsely positive on skin biopsy. Njaa *et al.* (2000) found positive staining for BVDV with IHC testing on four skin samples in animals acutely infected with a high dose of BVDV. The staining was, however, confined to a few small discrete foci in the stratum spinosum, with

little extension into follicular ostia, and was distinguishable from the more extensive staining in PI animals. Libler-Tenorio *et al.* (2003) found that BVDV antigen in inoculated calves was only present in lymphoid tissue and the intestinal mucosa and not in the skin. Ridpath *et al.* (2002) infected animals with six different isolates and could not detect virus in any of the skin biopsies. Hilbe *et al.* (2007) only detected positive staining results in the skin in PI animals and none in acutely infected animals. The summation was made that skin biopsies will rarely confuse PI animals with acutely infected animals and can be used as a reliable, fast, diagnostic tool to diagnose PI animals (Luzzago *et al.*, 2006).

Bovine viral diarrhoea virus antigen may also be demonstrated by the antigen capture enzyme linked immunosorbent assay (ELISA). There are several different ELISA's on the market that can specifically detect antigen (Brinkhof *et al.*, 1996; Sandvik 1999; Brock 1995; Hilbe *et al.* 2007). They all recognize antigenically conserved non-structural protein NS2-3 and are able to detect most if not all BVDV strains. The value of these tests lies in their good diagnostic performance, independence of cell culture and speed. After the research of the author had been finalised, the ELISA used to detect antigen in serum, was extended to also detect antigen on ear notch samples. This ELISA method on ear notch samples is comparable with the immunoperoxidase stain on ear notch biopsies and is also used to detect persistently infected animals. The HerdChek BVDV Antigen/Serum Plus Test Kit showed 100% accuracy in ring trials. The test is based on the detection of the robust E^{ms} (gp48) antigen in both serum and tissue and is highly stable (IDEXX Laboratories, ZUL. Nr. BGVV-B230 version 06-43860-02).

ELISA is a versatile diagnostic method to detect almost any immunoreactive molecule and can also be used to detect serum antibodies. Clinically healthy PI animals older than 2 months are nearly always negative for antibodies (Sandvik, 1999). The antibody ELISA method of testing is independent of cell culture; it is reliable, fast and can easily be applied for mass screening (Sandvik 1999; Brock 1995; Chu *et al.*, 1985, Hilbe *et al.*, 2007). The antibody ELISA diagnostic test may give a good indication if animals arriving at a feedlot had previous exposure to BVDV, most likely from a PI animal on the farm of origin.

CHAPTER TWO

STUDY PURPOSE AND OBJECTIVES

2.1 Purpose of the study

The prevalence of PI animals entering feedlots in South Africa is unknown. The IMP stain for BVD viral antigen on ear notch samples is regarded as a reliable, fast diagnostic tool to identify PI animals. The prevalence of PI animals entering the feedlots in South Africa will be known after this research.

If PI animals can be identified soon after arrival at a feedlot, they can be timeously removed to prevent spreading of virus to susceptible animals.

The farms of origin of PI animals can be located by the feedlots and the farmers can be advised on specific control measures for BVDV to reduce the prevalence of PI animals sold to feedlots.

The feedlots will gain financially due to a decrease in losses incurred from treatments, prolonged standing time and poor performance.

2.2 Objectives

1. To determine the prevalence of PI animals entering feedlots, by means of immunoperoxidase evaluation of skin biopsies.

2. To determine if PI animals are at higher risk of contracting respiratory disease in the feedlot than non-infected animals
3. To determine the reliability of the IMP technique by investigating non-specific staining or staining of few mononuclear cells with reevaluation and/or restaining with another color.
4. To determine the reliability of the IMP technique to identify PI animals in comparison with the antibody and antigen capture ELISA's.

CHAPTER THREE

MATERIALS, METHODS AND RESULTS

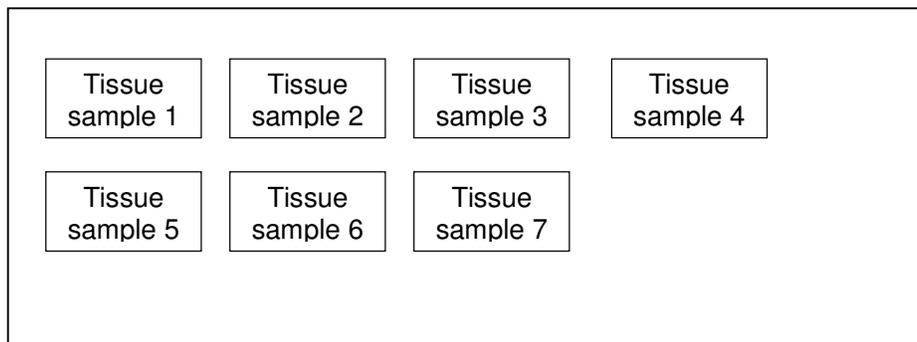
Ear notch biopsies were collected from calves at the point of entry into feedlots and from chronic poor doers, as well as from calves presented to the hospital pen for the first time from the following feedlots: Karan Beef, Mollevel, Taaiboschbult, Gysbertshoek, Hurland, and Sparta Beef. The calves selected at point of entry were unthrifty, thin, and potbellied, with a dull hair coat and poor looking, suspected to be persistently infected with BVDV. The chronic poor doers were animals which had received more than three treatments in the hospital pen and were placed on veld because of poor performance in the feedlot. Dead animals of this group were also sampled. A total of 1074 specimens were collected from first time entrants and chronic poor doers, and 616 from animals, pulled for respiratory disease, entering the hospital pen for the first time.

Ear notch samples, measuring 1cm by 0.5cm, collected with an ear tag applicator were fixed in 10% buffered formalin and presented to the laboratory for examination, within three to four weeks after collection. Ear notch samples were taken because of the ease of collection, but biopsy specimens could have been taken from any skin area, as no difference in distribution of BVDV antigen is present in skin samples (Thur *et al*, 1996).

3.1 Immunoperoxidase staining

Sections were cut and embedded in wax blocks by routine standard operating procedures at the immunohistochemistry laboratory at the Pathology section, Faculty of Veterinary Science, Onderstepoort. Seven specimens were placed in a single wax block as demonstrated in figure 3.1. Each animal could thus be identified individually and costs were minimised.

Figure 3.1 Wax block demonstration



Sections of 3-4 microns were cut from the wax blocks. The tissues were then mounted on pre-treated Superfrost Plus glass slides for immunoperoxidase staining. The slides were dried overnight in an oven at 58 degrees Celsius to enhance tissue section adhesion. Routine dewaxing and rehydration was carried out (10 minutes in xylol and 3 minutes in each of 100%, 96% and 70% alcohol according to Bancroft & Stevens, 1982, modified version). The ABC Vector Elite immunoperoxidase staining procedure was used (Vector Elite ABC kit from Merck, catalogue Number PK 6100) [Fig. 3.2]. After dewaxing and rehydration antigen was demasked according to the proteolytic enzyme digestion (Pronase) method.

Three percent (3%) hydrogen peroxide was used (in distilled water) for 5 minutes (to block endogenous peroxidases). Sections were rinsed twice, once in distilled water and once in phosphate buffered saline/bovine serum albumin (PBS/BSA) buffer for 5 minutes per rinse. The pronase (SIGMA Protease Type XIV) solution was prepared by adding 50mg of the pronase powder to 100ml of PBS/BSA buffer that had been heated to 37 degrees Celcius. The sections were incubated in the pronase/buffer solution in the oven at 37 degrees Celcius for 30 minutes. They were then rinsed in distilled water and then in PBS/BSA buffer for 5 minutes per rinse.

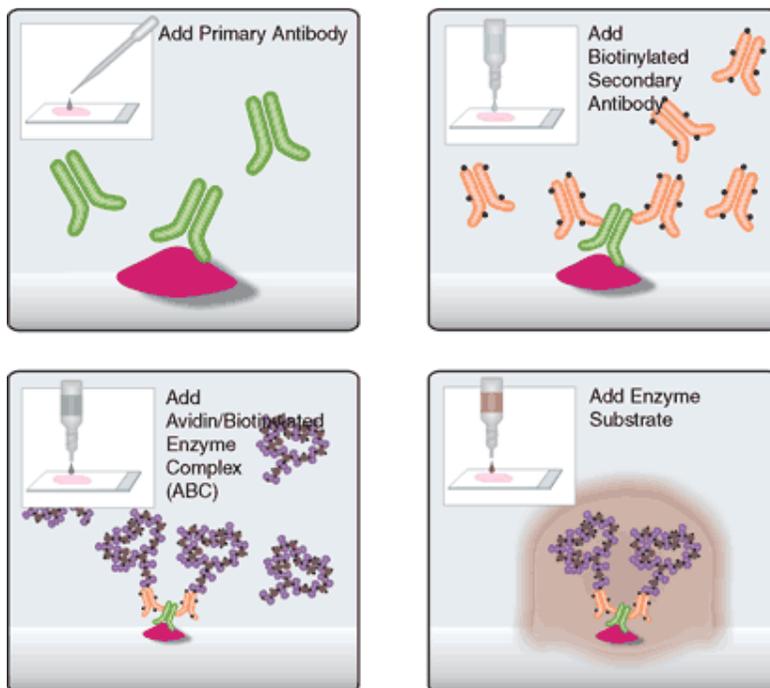
One drop of refrigerated normal rabbit serum was added to 10 drops PBS/BSA buffer, placed directly on sections and left for 20 minutes. The excess normal serum was gently shaken off the slides and was wiped gently around tissue sections. The primary monoclonal BVD antibody (Cornell University) was applied to all the appropriate sections at a dilution of 1:1000 and left to stain for one and a half hours on the bench at room temperature. Negative controls were prepared using only PBS/BSA buffer or an irrelevant monoclonal antibody. The sections were rinsed twice, once in distilled water and once in PBS/BSA buffer for 5 minutes each.

The secondary biotinylated antiserum (rabbit-anti-mouse antibody, diluted at 1:500) was applied on sections for 30 minutes and left at room temperature. The sections were then rinsed twice, with distilled water and then with PBS/BSA buffer for 5 minutes each. Peroxidase Conjugated Avidin was then applied (prepared according to manufacturer's instructions) for 30 minutes on the bench at room temperature. The slides were rinsed twice as described before. The sections were placed in a

DAB or NovaRED substrate (using a droplet method) for approximately 1 minute. As soon as clear positive staining was observed macroscopically on the positive control slide, all other slides were rinsed in a distilled water bath. The sections were then counterstained with haematoxylin for 3-4 minutes.

The sections were then rinsed under running tap water for 10 minutes to remove excess DAB/NovaRED substrate followed by routine dehydration through increased alcohol concentrations (70%, 96% and 100%) and xylol (Vector Laboratories, 2009). Sections were then mounted and coverslipped.

Figure 3.2 ABC Vector Elite immunoperoxidase staining procedure (Vector Laboratories www.vectorlabs.com)



3.2 BVD virus antibody ELISA

All reagents were allowed to reach room temperature (18°-25°C) and were mixed by gentle swirling or vortexing. Coated plates were used for the serum samples (ELISA flat bottom plates 446140) and the sample position was recorded on a worksheet. A 100 µl amount of sample diluent was added to each well (50 µl serum and 50 µl diluent). Twenty five µl of negative and positive controls was added in appropriate wells. Twenty five µl test samples were added to the remaining wells. The contents of the microwells were mixed by gentle tapping of the plate. The samples were incubated for 90 minutes at room temperature (18°-25°C) or overnight (12-18 hours) at 2°-8°C (in a refrigerator). Plates were tightly sealed or incubated in a humid chamber to avoid any evaporation. The liquid content of all wells was then aspirated into an appropriate waste reservoir. Each well was washed with approximately 300 µl of wash solution, 5 times. Liquid contents were aspirated after each wash. After the final aspiration, residual wash fluid from each plate was firmly tapped onto absorbent material. Plate drying between washes was avoided. A 100 µl amount of conjugate was then dispensed into each well. The samples were incubated for 30 minutes at room temperature, after which the washing was once again performed. A 100 µl amount of TMB substrate solution (3,3',5,5'-tetramethylbenzidine) was then dispensed into each well. The samples were incubated at room temperature in darkness for 10 minutes with timing started after the first well was filled. A 100 µl amount of stop solution (a proprietary solution used to terminate the peroxidase/TMB reaction) was then dispensed into each well to stop the reaction, in the same order as the substrate solution was added.

The spectrophotometer (ELX800, Universal micro plate reader, Bio-tek Inc.) was blanked on air. The absorbance of samples and controls were measured and recorded at 450 nm or using dual wavelengths of 450 nm and 650 nm. The results were then calculated. For the assay to be valid the difference between the positive control mean and the negative control mean had to be greater than or equal to 0.150 optical density (OD). In addition the negative control mean had to be less or equal to 0,250 OD (IDEXX Laboratories Zul. Nr. BGVV-B233, version 06-44000-02). The BVD virus antibody ELISA's were performed by Golden VetLab, now IDEXX South Africa, Woodmead Willows Office Park, 19b Morris Street, Woodmead.

3.3 BVD virus antigen capture ELISA

The first step in this procedure was sample dilution (50 µl serum/ear notch tissue soaking buffer IDEXX diluted 1 to 10 with distilled water and 50 µl diluent). All reagents were allowed to equilibrate at room temperature (18°-25°C) before use. Reagents were mixed by gentle swirling or vortexing. Coated plates (coated with anti-E^{ms} monoclonal antibodies) were obtained (part of the IDEXX BVDV Antigen Test Kit) and the sample position was recorded on a worksheet.

Fifty µl of the detection antibodies, which is part of the IDEXX BVDV Antigen Test Kit, was added to each well with a multichannel pipette. Then 50 µl negative and positive controls were added in the appropriate wells. Fifty µl test samples were then added to the remaining wells. The contents of the microwells were mixed by gentle tapping of the plate. The samples were then incubated for 2 hours at 37°C or overnight at 2°-8°C in a refrigerator. The plates were tightly sealed or incubated in a

humid chamber to avoid evaporation. The liquid contents were then aspirated into an appropriate waste reservoir. Each well was then washed with approximately 300µl of wash solution, five times. The liquid contents were aspirated after each wash. Following the final aspiration, residual wash fluid was firmly tapped from each plate onto absorbent material. Plate drying was avoided between washes. No traces of blood were left on the walls of the wells. A 100 µl amount of conjugate (Horseradish Peroxidase (HRPO) conjugate) was then dispensed into each well. The samples were then incubated at room temperature (18°-25°C) for 30 minutes. Liquid was then again aspirated and the samples washed 5 times as described above. A 100 µl amount of TMB substrate solution was then dispensed into each well. The samples were incubated for 10 minutes at room temperature in darkness. Timing began after the first well was filled. A 100 µl amount of stop solution was dispensed in each well to stop the reaction, in the same order as the substrate solution was added. The spectrophotometer was blanked on air. The absorbance of samples and controls were measured and recorded at 450 nm or using a dual wavelength of 450 nm and 650 nm. The results were then calculated. For the assay to be valid, the difference between the positive control mean and the negative control mean had to be greater or equal to 0.150 optical density. In addition the negative control mean had to be less or equal to 0.250 OD. (IDEXX Laboratories, Zul. Nr. BGVV-B230 version 06-43860-02). The BVD virus antigen capture ELISA's were performed by Golden VetLab, now IDEXX South Africa, Woodmead Willows Office Park, 19b Morris Street, Woodmead.

3.4. RESULTS

3.4.1 Immunoperoxidase staining

In total 1690 ear notch biopsies were received for processing and immunoperoxidase staining. Of these 49 samples (2.9%) were positive for BVDV antigen with immunoperoxidase staining. One thousand and seventy four (1074) samples were from calves entering feedlots and chronic poor doers. The remaining 616 samples were from calves entering the hospital pen for the first time because of respiratory disease. Forty three samples (4%) stained positive in the 1074 group (calves entering feedlots or chronic poor doers). Only 6 out of 616 (1%) stained positive in calves entering the hospital pen with respiratory disease for the first time. Cases that showed granular DAB or NovaRED coloured staining in the keratinocytes of the epidermis, epithelial cells in hair follicles, smooth muscle cells and fibroblasts were regarded as positive (Njaa *et al.*, 2000). Figures 3.3, 3.4, 3.5 and 3.6 demonstrate the positive staining observed with DAB and NovaRED.

Figure 3.3 DAB positive staining in the keratinocytes of the epidermis (top arrow) and hair follicle epithelium (middle and bottom arrow) of a PI calf x40

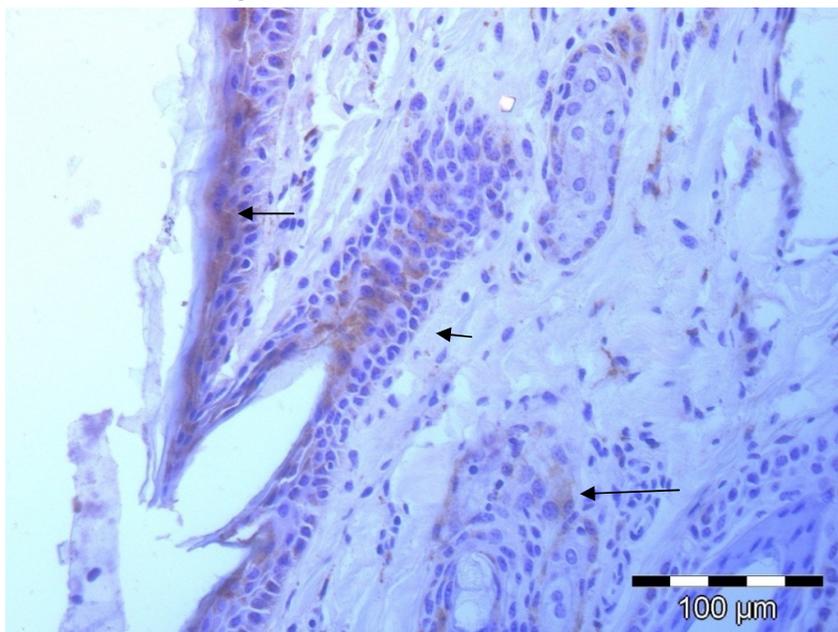


Figure 3.4 NovaRED positive staining in the epidermis (top arrow) and hair follicle epithelium (bottom arrow) of a PI calf x40

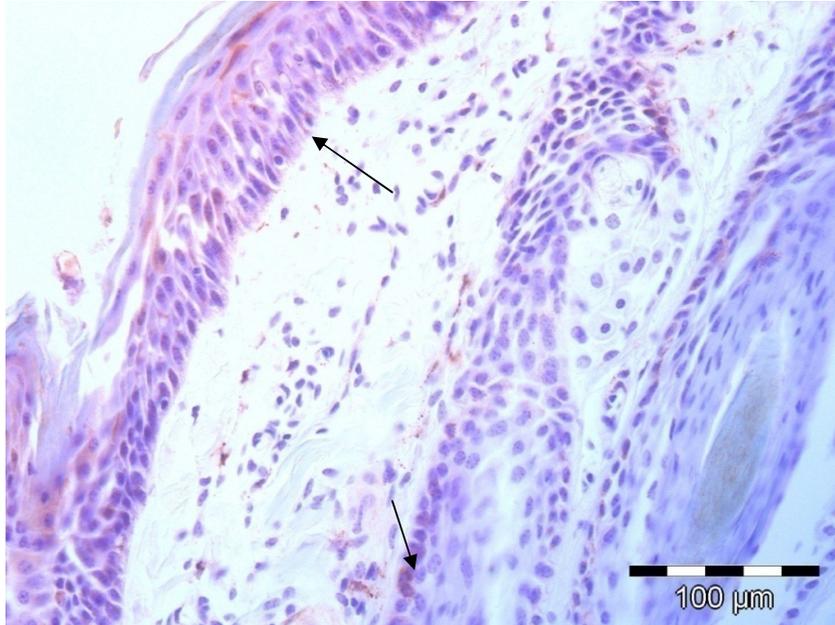


Figure 3.5 NovaRED positive fibroblasts in the dermis (arrows) of a PI calf x40

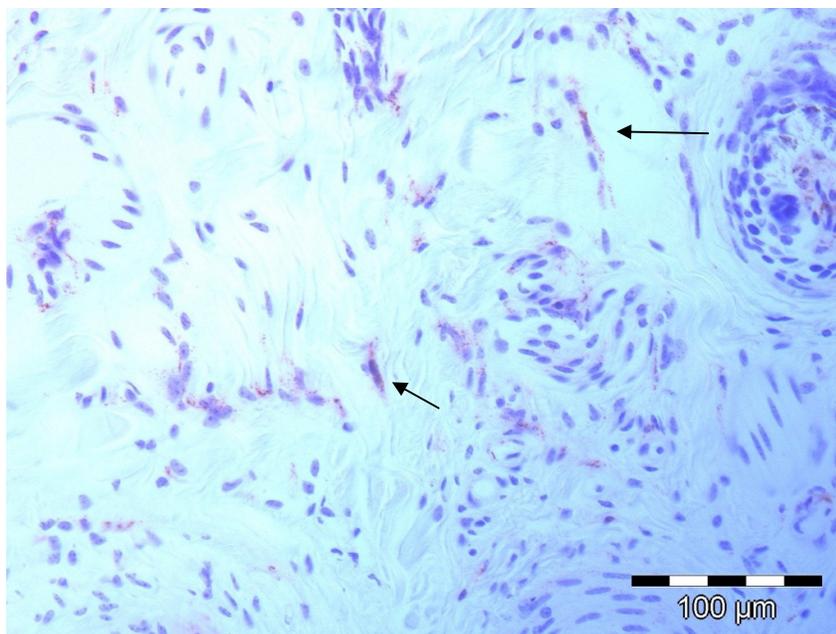
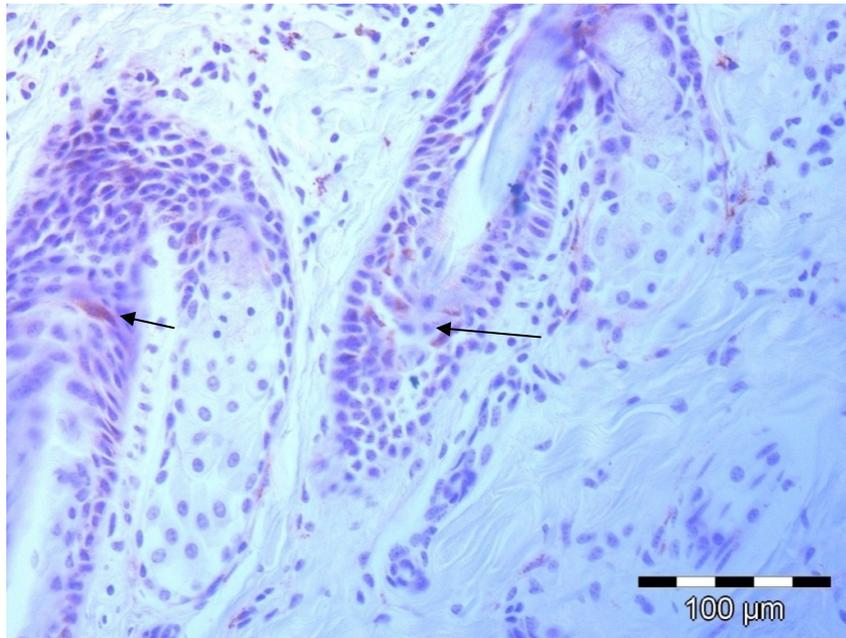


Figure 3.6 NovaRED positive staining of hair follicle epithelium (arrows) in a PI calf x40

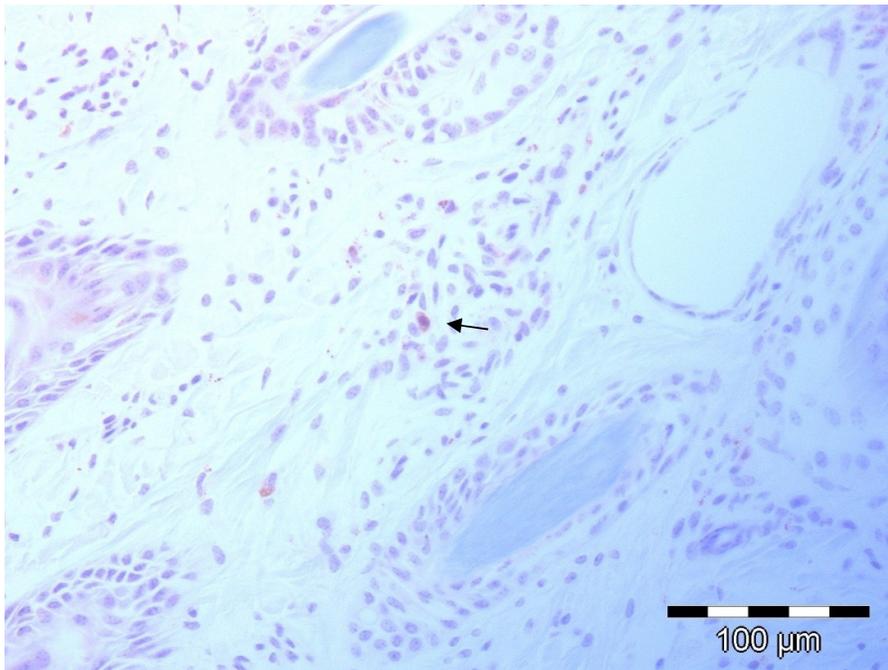


All samples were evaluated twice as abnormally high numbers of cases appeared to stain positive in one group with the first evaluation. In this group from Taaiboshbult, 75 out of 154 cases apparently stained positive. There was concern regarding this situation and the staining patterns were investigated. The conclusion was reached that a high number of cases showed non-specific positive staining (*vide infra*). After re-evaluation, as described below, only 12 positive cases were identified.

Non-specific staining was defined as non-granular DAB or NovaRED staining visible mainly in spindle and round cells in the dermis [Fig. 3.7]. In the calves under discussion it was noted that only spindle-shaped cells and round cells stained

positive and no positive staining was evident in keratinocytes, hair follicle epithelial cells or smooth muscle cells.

Figure 3.7 NovaRED non-specific positive staining of mast cells in the dermis (arrow) x40

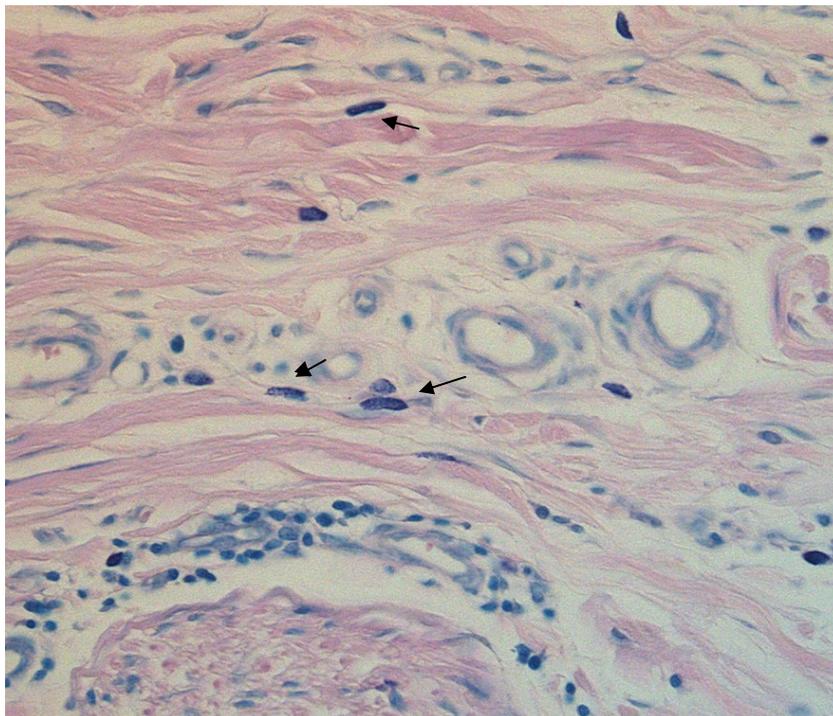


Positive staining of mast cell granules was suspected (Personal consultation with Dr Clift, Pathology section, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort) and after staining of sections with the Ziehl Neelsen (ZN) stain this was confirmed [Fig. 3.8] (the cells that showed positive staining with DAB or NovaRED showed positive staining with Ziehl Neelsen staining as well). Most of the bovine mast cells in the skin were spindle-shaped, which was

confirmed with the Ziehl Neelsen stain. Mast cell granules stain blue/basophilic with the Ziehl Neelsen stain (University of Rochester, 2007

www.urm.rochester.edu/Path/zqu/Stains Manual/index.html).

Figure 3.8 Ziehl Neelsen basophilic granular staining of spindle-shaped mast cells in the dermis (arrows) x40



Mast cells stained more regularly positive with the DAB colour marker in comparison with NovaRED. The majority of the non-specific cases were stained with DAB. Another problem with using DAB as colour marker was that the colour pigment was sometimes difficult to distinguish from melanin pigment in the skin (yellow to golden colour). The researcher preferred NovaRED as colour marker as it cannot be confused with another colour, due to its unique brick red colour.

3.4.2 BVD virus antibody ELISA and BVD virus antigen capture ELISA

Only ten of the animals, which originally tested positive with the immunoperoxidase stain, had serum samples available to test for BVDV antibody and antigen. The results were as follows with positive indicating the presence of antibodies or antigen and negative the absence thereof. Values above 0.200 for antibodies are positive.

Table 3.1 BVD virus antibody and antigen capture ELISA results

Sample number	Antibody ELISA titre values	Antibody ELISA result	Antigen ELISA result
1.	0.271	Positive	Positive
2.	0.111	Negative	Positive
3.	0.326	Positive	Positive
4.	0.149	Negative	Positive
5.	0.114	Negative	Positive
6.	0.084	Negative	Negative
7.	0.412	Positive	Negative
8.	0.473	Positive	Negative
9.	0.585	Positive	Negative
10.	0.223	Positive	Positive

Six of the ten animals, which showed positive staining with the immunoperoxidase stain, tested positive for viral antigen with the ELISA. Persistently infected animals with BVDV will always be antigen positive, as they are consistently viraemic. This result indicated that animals 6, 7, 8 and 9 were not persistently infected with BVDV.

These cases were re-evaluated, and as discussed later, they showed non-specific positive staining in mast cells, leading to the false positive diagnosis.

A persistently infected animal will not have antibodies against the strain of BVDV, which infected it *in utero*. Three of the six animals, which tested positive for viral antigen, did not have antibodies against BVDV, confirming their PI status. If an animal is however infected with a different strain of BVDV after birth, they will develop antibodies to that strain. This explains why three animals, positive for viral antigen and positive PI animals on IMP stain, tested positive for antibodies against BVDV.

CHAPTER FOUR

DISCUSSION

BVDV immunoperoxidase staining on ear notch biopsies is considered a fast and accurate diagnostic tool for persistently infected animals (Thur *et al.*, 1996; Houe *et al.*, 2006; Luzzago *et al.*, 2006; Hilbe *et al.*, 2007). The overall prevalence of persistently infected calves entering the feedlot in this study was 2.9%, which is much higher than the estimate of Ishmael, 2003, who predicted an intake of 0.5% by the industry. The estimate was based on the assumption that 1% persistently infected animals are born into infected herds per year and half of the animals will die before weaning. Less than a half percent of animals will thus be available to enter feedlots due to the early deaths of PI animals. Non-published data from research performed by Schultheiss, Thompson and Henson in July 2004, indicate a prevalence of PI animals of 0.56% in a single feedlot. Samples were randomly collected from calves entering the feedlot and each animal had an equal chance to be selected. In total 2994 out of 20 000 animals were tested. The prevalence corresponds with that of Ishmael (2003) (Personal communication with Dr Willem Schultheiss, Intervet Schering-Plough Animal Health, 2010). The samples in this study were however taken from a selected population of calves, which were visually suspect calves and chronic poor doers and cannot be directly compared to the other studies. Some feedlots may receive large numbers of animals from a potentially large number of infected herds, increasing the possible intake of PI animals. Loneragan *et al.* (2005) states that when one PI animal is identified in a herd, the

herd likely contains more than one, and that it is thus possible that PI animals entering a feedlot are similarly clustered by herd of origin. A higher percentage of positive animals may thus be present in a specific feedlot if compared to a single infected herd where approximately 1-2% calves born are persistently infected.

The samples were collected mainly by feedlot personnel, and distinctions were not made between animals entering the feedlot for the first time and chronic poor doers. It is thus not possible to determine how many suspected PI first time entrants and how many chronic poor doers were sampled. It was however clear from the samples received, that feedlot personal preferred sampling chronic poor doers in comparison to first time entrants. Selection of first time entrants was based on their physical appearance including being pot-bellied, unthrifty and thin calves. The feedlots were specifically interested in the possible causes resulting in a chronic poor doer as it is an important problem in the feedlot industry, resulting in financial losses. From this the conclusion is made that the likelihood of a PI animal to become a chronic poor doer in a feedlot is increased, which may also have played a role in the higher prevalence of 2.9% in this study. Loneragan *et al.* (2005) found that PI animals were more likely to become chronically ill or die. In the group of animals entering the hospital pen for the first time (collected separately at the end of the trial), only 1% were persistently infected on immunoperoxidase staining.

It was proposed by the researcher that being persistently infected would increase the likelihood to contract a respiratory illness early after admittance, due to the immunosuppressive nature of the disease. Other factors causing respiratory disease in cattle in feedlots include the time of year (more prevalent in winter), extremely

dusty conditions, stressors such as over stocking, aggressive pen mates, rain and mud, wind, poor adaptation, non-vaccinated (not pre-treated) animals and respiratory disease following acidosis. Only 1% of animals entering the hospital pen for the first time, were persistently infected with BVDV and an increase in respiratory disease in PI animals, shortly after admittance, does not seem to be the case with the data collected. Schultheiss, Thompson and Henson found no specific evidence of an increase in pneumonia in PI calves, but the presence of a PI animal in a pen increased the risk of the other calves in the pen to contract pneumonia three fold (Personal communication with Dr Willem Schultheiss, Intervet Schering-Plough Animal Health, 2010). This also corresponds with the literature as discussed in the introduction. Loneragan *et al.* (2005) found an increase in respiratory tract disease in PI's. The authors also found the incidence of respiratory tract disease 43% greater in cattle with an opportunity of direct contact with a PI animal. They also required more treatment for respiratory disease compared to cattle not exposed to a PI animal.

The reliability of immunoperoxidase staining to diagnose persistently infected animals was also tested in this study. One of the most important findings was the possibility to diagnose an animal incorrectly as persistently infected with BVDV because of non-specific staining. Investigation into this problem was done after an inappropriate number of positive cases were diagnosed. Non-specific positive staining was observed in round mast cells as well as spindle-shaped mast cells. As the candidate becomes more used to evaluate these sections, it becomes clear when mast cells are staining positive as the granules have a distinctly different shape from the granular virus staining. All sections diagnosed as positive were re-evaluated to exclude any false positives in the final results. Although non-specific

staining was observed with both stains, the vast majority was seen with DAB staining. It is not exactly certain why mast cell granules stain positive with the BVDV antibody. This phenomenon is not seen in every negative case and mast cells granules do not always take up stain. The possibility of over staining must also be considered, as many of the affected sections showed colour changes in the connective tissue and cartilage indicative of overstaining.

All positively diagnosed animals had to have positive staining in keratinocytes of the epidermis (stratum basale and spinosum), hair follicle epithelium, smooth muscle cells of blood vessels and spindle-shaped cells in the connective tissue of the dermis (fibroblasts). If these parameters are taken into consideration and if cases are carefully examined, immunoperoxidase staining can be considered as a fast, reliable and cost effective tool to identify persistently infected animals. Cornish *et al.* (2005) found that the immunoperoxidase stain detected 100% of persistently infected calves. The monoclonal antibody used in the immunoperoxidase staining method was received from Cornell University in the United States of America, and was specifically developed for research purposes and is considered to be of the highest quality. All samples in this research project were stained therewith.

One of the limitations of this study was the inability to collect serum samples from all animals sampled. Only 10 PI animals, diagnosed with BVDV immunoperoxidase staining, had serum samples available for testing. Six calves were positive for BVDV antigen (consistently viraemic) and of these 3 had antibodies to BVDV (table 3.1). The IDEXX HerdCheck BVDV Antibody Test Kit has a high specificity and sensitivity and detects BVDV types I and II antibodies (IDEXX Laboratories ZUL. Nr. BGVV-

B233, version 06-44000-02). A persistently infected animal will not have antibodies against BVDV as the immune system recognises the virus as part of the animal's system, unless the animal was infected by another strain of the virus after birth (McClurkin *et al.*, 1984). Animals infected *in utero*, after the immune system is competent, will have antibodies against this viral infection. Therefore the BVD virus antibody ELISA cannot be used on its own to identify a persistently infected animal. In this study three of the six persistently infected animals showed the presence of antibody, indicating infection by a different strain of virus after birth. This is compatible with the literature that a persistently infected animal may be infected with a different strain of the virus and may develop antibodies to that specific strain. They will however be antibody negative towards the strain which caused the persistent infection.

A persistently infected animal will have a positive result with the BVD virus antigen capture ELISA as it is persistently viraemic. An acutely infected animal will however also test positive, and the test, on serum samples, is thus not diagnostic to confirm PI status. The test is based on the robust E^{ms} (gp48) antigen, which is consistently present in large amounts in both serum and tissue, making it easy to detect with the test kit. This antigen is highly stable leading to reliable results even after long storage (IDEXX Laboratories, ZUL. Nr. BGVV-B230, version 06-43860-02). Four animals were negative for antigen and positive for antibody indicating that they were not persistently infected. Re-evaluation of the immunoperoxidase sections indicated that all four of these were incorrectly diagnosed originally as positive due to the presence of non-specific staining. Based on the information obtained in this study the immunoperoxidase method, is therefore a good and reliable tool to diagnose

persistently infected animals, and corroborate the findings of Thur *et al.* (1996); Cornish *et al.* (2005); Luzzago *et al.* (2006); Houe *et al.* (2006) and Hilbe *et al.* (2007). However, the pathologist evaluating these sections must be able to differentiate clear positive staining from non-specific staining according to the advised parameters.

Too few skin samples were accompanied by serum samples to statistically compare the IMP stain method with the ELISA antibody and antigen determinations. In general the IMP method is more cost effective as only one test needs to be performed in comparison with both ELISA's. Currently the cost for one animal's IMP test is approximately equal to the cost of one of the ELISA's.

CHAPTER FIVE

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