BOVINE PESTIVIRUS DISEASE :

AN INVESTIGATION OF A SEVERE OUTBREAK OF BOVINE VIRAL DIARRHOEA VIRUS INFECTION IN CALVES IN NEW ZEALAND.

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A THESIS PRESENTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF VETERINARY STUDIES

IN

INFECTIOUS DISEASES

AT MASSEY UNIVERSITY, PALMERSTON NORTH, NEW ZEALAND.

OCTOBER 2000

ERRATA

The following are corrections to the text since the thesis went to print :-

Page no. xi, line12 - **VIAS** should be "Victorian Institute of Animal Science"

Page no. 66, line1 - "Kruskat" should be "Kruskal"

Page no. 26, line8 - "Peyers patches" should be "Peyer's patches

Page no. 38, line 4 - "thosand" should be "thousand"

Page no. 101, line 11 - "severe pathology" should be "severe pathological changes"

ABSTRACT

An outbreak of bovine pestivirus disease, in which there was high mortality (37%) in 102 calves, was investigated. It was postulated that the severity of the outbreak may have been due to the presence of a highly virulent strain of bovine viral diarrhoea virus.

Nine calves from the field outbreak were transported to Massey University for detailed clinical, post-mortem and laboratory examination. Samples were also submitted from a further three animals on the farm.

The results of immunological and virological studies indicated that seven calves had acute bovine viral diarrhoea virus infection and five calves had mucosal disease. Although the mucosal disease cases showed more severe clinical signs, lesions were widespread in both groups.

A non-cytopathic bovine viral diarrhoea virus isolate from one calf was used as the challenge virus in a transmission experiment designed to investigate the pathogenicity of this strain. The 11 calves used in this experiment comprised of four unvaccinated, challenged calves, four vaccinated calves (two challenged, two in-contact), two unvaccinated, in-contact calves and a control (neither vaccinated nor in-contact). The experiment took place over a month, allowing multiple clinical examinations and sampling procedures to be carried out before necropsy.

The challenge virus caused mild disease, with lesions similar to those reported in experiments in which Type 1 bovine viral diarrhoea virus isolates were used. Following experimental challenge, virus was not recovered from the calves, but a serological diagnosis of bovine viral diarrhoea virus infection was made by demonstrating a greater than fourfold rise in titre of bovine viral diarrhoea virus antibody in all challenged calves. There were only minor changes in haematological indices in challenged calves. The six challenged calves showed two distinctive lesions in intestinal sections. These were crypt necrosis (of glands of Lieburkuhn) and cryptal prolapse (herniation of crypts into the submucosal site of Peyer's patches depleted of lymphocytes). In the disease outbreak, these lesions were only observed in the mucosal disease cases.

Focal haemorrhages at sites of lymphocytic nodules were found in the nasal cavity of all challenged and vaccinated calves in the transmission experiment, but not in the unvaccinated, in-contact calves or the control calf. These lesions have not been reported in natural infections.

Vaccination was only partially protective, and there was evidence of spread of bovine viral diarrhoea virus infection to one vaccinated, in-contact calf. Scoring of histological lesions allowed a measurement of the effect of vaccination. There was a 60% reduction in the total histological lesion score in the four vaccinated calves (two challenged, two in-contact) when compared with the four unvaccinated, challenged animals.

It was concluded that the high mortality seen in the calves in the field outbreak was due to mucosal disease, and that this was consequential to a high infection rate in the dams during pregancy at a time when the foetuses were at risk of becoming persistently infected (45-125 days of gestation).

The pathological "fingerprint" for bovine viral diarrhoea virus infection was found to be the concomitant finding of three lesions at necropsy. Firstly, erosive lesions in the squamous epithelium of the upper alimentary tract. Secondly, catarrhal enteritis, with the distinctive and characteristic microscopic lesions of crypt necrosis and cryptal prolapse. Thirdly, lymphoid tissue lesions, especially lymph node enlargement, lymphoid depletion and inflammation of Peyer's patches.

Despite the difficulties in pathotyping the challenge virus in the transmission experiment, there was little evidence that it was a Type 2 strain. Genetic typing of this virus, by sequencing of polymerase chain reaction products, would be useful in determining its place in the phylogeny of bovine pestiviruses.

ACKNOWLEDGEMENTS

Thanks are due to the Institute of Veterinary, Animal and Biomedical Sciences for providing the facilities used, as well as some financial assistance (Post Graduate Research Fund).

The two major sponsors for this study were The Dairy Cattle Society of the New Zealand Veterinary Association and Merial Ltd, New Zealand. Their support was greatly appreciated.

Many thanks must go to my three supervisors for their guidance, encouragement and friendship throughout the project. I would like to thank my Chief Supervisor, Dr. Colin R. Wilks for his sharing of knowledge on bovine pestivirus disease and also his enthusiastic approach to infectious diseases in general. I wish to thank Dr Joanne Meers for her assistance in the financing and planning of my thesis, as well as her expert guidance in the virological aspects of the work. Thanks also to Dr. Maurice R. Alley for his diligent assistance with the large pathology sections in my thesis.

The nature of this study lead to the widespread use of technical assistance. Not only was this specialist expertise most appreciated, but the cheerful approach of those concerned helped make the study a most enjoyable one. Thanks to Faris Sharpe (necropsy), Pam Slack and Pat Davey (histo. slide preparation), Laurie Sandel (virology), Kylie Walker and Magda Gwozdz (bacteriology) and Barbara Adlington and Shirley Calder (parasitology).

I would like to kindly thank the farmer, Neville Ardern, and his veterinarian, Dr. Jon J. Le Gal, for providing me with the material that allowed the start to my investigation.
A thank you is also due to the manager of Riverside Farm, Neil Kilmuster, for his assistance with the selection of calves for the transmission experiment.

Finally, I would like to thank my family for their support during this project, especially my fiance, Julie Vickery, for her encouragement during the long and arduous writing-up procedure.

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LIST OF ABBREVIATIONS

AI	artificial insemination
ATV	antibiotic-trypsin-versene
AV-4	averonite-four
BAHL	Batchelor Animal Health Laboratory
Belu	bovine embyonic lung
BDV	border disease virus
BRS	bovine respiratory syncitial
BRSV	bovine respiratory syncitial virus
BVD	bovine virus (or viral) diarrhoea
BVDV	bovine virus (or viral) diarrhoea virus
CAHL	Central Animal Health Laboratory
CNS	central nervous system
CP	cytopathic
CPE	cytopathic effect
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EBL	enzootic bovine leucosis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
E-MEM	Eagles minimum essential medium
ES	equine serum
FEC	faecal egg count
GIT	gastro-intestinal tract
GM	growth medium
H&E	haemotoxylin and eosin
IBR	infectious bovine rhinotracheitis
IBRV	infectious bovine rhinotracheitis virus
IgG	immunoglobulin G
IP	immunoperoxidase
ISCOMs	immune-stimulating complexes
MCF	malignant catarrhal fever
MD	mucosal disease
MDBK	Madin Darby bovine kidney
MLV	modified-live virus
MM	maintenance medium
NADL	National Agriculture Department Laboratory
NCDI	National Centre for Disease Investigation
NCP	non-cytopathic
NY	New York
NY-1	New York-one
NZ	New Zealand
PBS	phosphate buffered saline
PCR	polmerase chain reaction
PI	persistently infected
p.i.	post-infection
PI3	parainfluenza-3
Pp	Peyer's patch

PSK	penicillin-streptomycin-kanamycin
RNA	ribonucleic acid
SFV	swine fever virus
SN	serum neutralisation
SNT	serum neutralisation test
SPF	specific pathogen free
$TCID_{50}$	tissue culture infective dose (50%)
TPB	tryptose phosphate broth
UAT	upper alimentary tract
URT	upper respiratory tract
USA	United States of America
VIAS	Veterinary Institute of Agricultural Science
WBC	white blood cell

CHAPTER ONE

REVIEW OF THE LITERATURE

INTRODUCTION

Acute enteric disease caused by bovine pestivirus was first described more than 50 years ago (Olafson *et al.*, 1946). Since that time, it has become recognised as an important virus in cattle throughout the world, causing a wide range of clinical signs (Ames, 1986 ; Brownlie *et al.*, 1987). Today, this virus is considered a primary pathogen of the bovine enteric, respiratory, reproductive, and immune systems, with epidemiological data clearly indicating that the virus induces economically significant disease in cattle of all ages (Bolin, 1996).

1.1 THE VIRUS

Bovine virus diarrhoea virus (BVDV) belongs to the genus *Pestivirus*, along with swine fever (or hog cholera) virus of pigs (SFV) and Border disease (or hairy shaker disease) virus of sheep (BDV) and was originally placed in the family *Togaviridae* (Westaway *et al.*, 1985). However, it has now been reclassified as a member of the *Flaviviridae* family following studies on the morphology and morphogenesis of the virus (Collet *et al.*, 1988 ; Bielefeldt Ohmann, 1990). Using electron microscopy, the virus is found to be small, ranging between 45-60 nm, and although mostly spherical, is pleomorphic. The virion consists of a core particle of 20-25 nm which is dense or semi-dense, and may be isometric or hexagonal. A membranous envelope which is smooth and bilaminar surrounds this core. This contrasts with the closely related swine fever virus which has surface projections on the envelope (Bielefeldt Ohmann, 1990).

The virus replicates in the cytoplasm of the cell, with assembly and maturation of virus particles occurring in membrane-bound vesicular organelles found in the region of the Golgi apparatus or endoplasmic reticulum (Bielefeldt Ohmann, 1990). Release of the virus occurs when the cell finally lyses or via exocytosis. 'Budding' of the cell wall, as

replication, as well as in morphology, were the first indications that the virus bore a closer resemblance to members of the *Flaviviridae* family than the *Togaviridae* family, and subsequent sequencing of the genome confirmed its place here. A description of the taxonomic position of BVDV is given by Horzinek (1990).

The genome of BVDV is an infectious single strand of RNA, with positive polarity (Hafez and Liess, 1972; Renard *et al.*, 1985). The genome is made up of 12578 nucleotides, which code for a number of structural and non-structural proteins as well as glycoproteins. Based on their genetic structure, all strains of BVDV fall into one of two groups :-

- Type I - includes most of the traditional strains worldwide.
- Type II - the new more virulent strains, seen mainly in North America.

Recent genetic typing of pestiviruses in N.Z. revealed that all viruses obtained from cattle were characterised as BVDV Type I, genetically close to the NADL strain (Vilcek *et al.* , 1998). Type II viruses are further discussed in section 1.4.6.

All strains of BVDV show some degree of serological cross reactivity, as do all three members of the pestivirus genus (Plant *et al.*, 1973; Bolin *et al.*, 1988). Monoclonal antibody analyses have demonstrated that BVDV isolates can be segregated into a number of different groups, and that both the cytopathic and noncytopathic biotypes exist as a number of different strains (Bolin *et al.*, 1988). Analysis of the gene sequences of a number of pestivirus isolates has confirmed the grouping established using monoclonal antibodies (Edwards and Paton, 1995). There is division into four clusters; BVDV Type I, BVDV Type II, SFV and BDV (Ridpath *et al.*, 1994, Paton *et al.*, 1995).

The wide antigenic diversity among BVDV is considered to be one of the reasons that vaccines are not protective against all strains of the virus (Bolin, 1996).

The virus is inactivated by lipid solvents, such as ether (Hermodson and Dinter, 1962), flavines, such as acriflavine (Brinton, 1980), certain enzymes, like trypsin (Hafez and Leiss, 1972) and various disinfectants such as phenols and chlorhexidine (Duffel and Harkness, 1985). Survival time for the virus varies with temperature. The virus is rapidly inactivated in one hour at 56 ^{O}C (Taylor *et al.*, 1963) while at 37 ^{O}C loss of viability occurs by 5 days (Tanaka *et al.*, 1968). At room temperature (20 ^{O}C) BVDV

viability occurs by 5 days (Tanaka *et al.*, 1968). At room temperature (20 ^{O}C) BVDV can be successfully isolated from blood for at least 5 days after collection (Rae *et al.*, 1987), whereas if refrigerated (4 ^{O}C) the virus is stable for at least 35 days (Tanaka *et al.*, 1968). In frozen tissues the virus is viable for 6 months at -20 ^{O}C (Ssentengo, 1978) and up to 16 months at -40 ^{O}C (Golo, 1989). The virus is stable over a pH range of 5.7 to 9.3, with a maximum stability at 7.4 (Hafez and Liess, 1972). The virus is readily inactivated by ultraviolet radiation and so survival in the environment does not generally exceed 14 days (Duffel and Harkness, 1985).

1.2 THE BIOTYPES OF BVDV

1.2.1 Laboratory definition and pathogenicity.

Bovine viral diarrhoea viruses exist as cytopathic (CP) or non-cytopathic (NCP) biotypes. This refers to the effect of each biotype on cells grown in the laboratory in cell-culture systems. NCP viruses produce little, if any, visible cytopathic change in cell cultures, and infected cells generally appear normal. In contrast, CP viruses cause cellular vacuolation and cell death. Three tissue culture passages are considered necessary to designate an isolate as either CP or NCP (Deregt and Loewen (1995). This laboratory classification into CP or NCP is not indicative of their ability to cause disease. The NCP biotype is considered to be the more important, and has been described as 'the genuine pestivirus' by Nettleton and Entrican (1995). It is the main form circulating in cattle populations, and is the biotype that has been shown to cross the placenta, invade the foetus, and set up the persistent infection so crucial for successful virus spread (Nettleton and Entrican, 1995). The NCP biotype is involved in both mild (type 1) and severe (type 2) diseases, as well as congenital disease. In contrast, the CP biotype is usually only involved in causing mucosal disease (MD) through superinfection of the persistently infected calf. An animal dying of MD often yields 'pairs' of biologically cloned CP and NCP viruses, indicating that the CP biotype may have arisen by mutation from the NCP virus within the persistently infected animal. The CP virus does not produce persistently infected animals, so this biotype is unable to spread to the next generation (Brownlie *et al.*, 1989).

Since both biotypes are always present in MD, viral isolates from these cases show cytopathic effect in cell cultures. Because MD cases show dramatic clinical signs and are invariably fatal, the CP biotype has, until recently, attracted much more attention than NCP isolates (Nettleton and Entrican, 1995).

1.2.2 Tissue tropism.

The two biotypes have been shown to have different tissue tropism (Clark *et al.*, 1985):-

- CP virus has a greater affinity for intestinal tissues, being readily isolated from rumen, reticulum, small intestine, mesenteric lymph node, Peyers patch and colon.
- NCP virus is more commonly isolated from blood (including serum) and blood-rich organs such as nasal cavity, lung, liver, kidney and spleen.

1.2.3 Serology and Molecular Biology.

The two biotypes are not distinguishable serologically. However, at a molecular level, the biotypes are different, as shown by comparison of the final protein products and their functions (Collett *et al.*, 1988). Nettleton and Entrican (1995) reviewed the subsequent work which revealed that the major non-structural protein produced in cells infected by NCP viruses is p125. In cells infected with CP virus this protein is cleaved to release an immunodominant p80 protein, along with p125 and p54 (Figure 1.1). The p80 protein is important because it is a highly conserved and immunogenic protein, with all CP-infected animals having high levels of antibody against it. At the present time, expression of p80 is the only apparent molecular marker of cytopathogenicity (Pocock *et al.*, 1987; Meyers *et al.*, 1991). It has been shown to be a proteinase involved in polyprotein processing (Wiskerchen and Collett, 1991). It has been postulated that p80 cleaves and destroys critical cellular proteins, whereas p125 does not. The other subunit that can be produced in CP viruses is the p54 protein. However this is variable between isolates, and antibodies against it cannot be detected readily in animals infected with CP virus (Nettleton and Entrican, 1995).

Further studies have involved investigation of 'pairs' of NCP and CP from individual animals. Insertion sequences have been found in the area of the genome encoding the p54. A ubiquitin-encoding insertion sequence was found in a CP BVDV isolate but not in its 'paired' NCP biotype, and it was proposed that recombination between viral and cellular RNA is responsible for the development of the CP virus (Meyers *et al.*, 1991). Of additional interest in this study was the observation that there was greater sequence homology between 'pairs' than with the NADL strain, supporting the hypothesis that the CP virus originates from NCP virus within an individual animal. There was also evidence that these mutations happen in more than one way. That is, the generation of CP virus could be due to recombination between cellular and viral sequences, but also by rearrangement of viral sequences (Nettleton and Entrican, 1995).

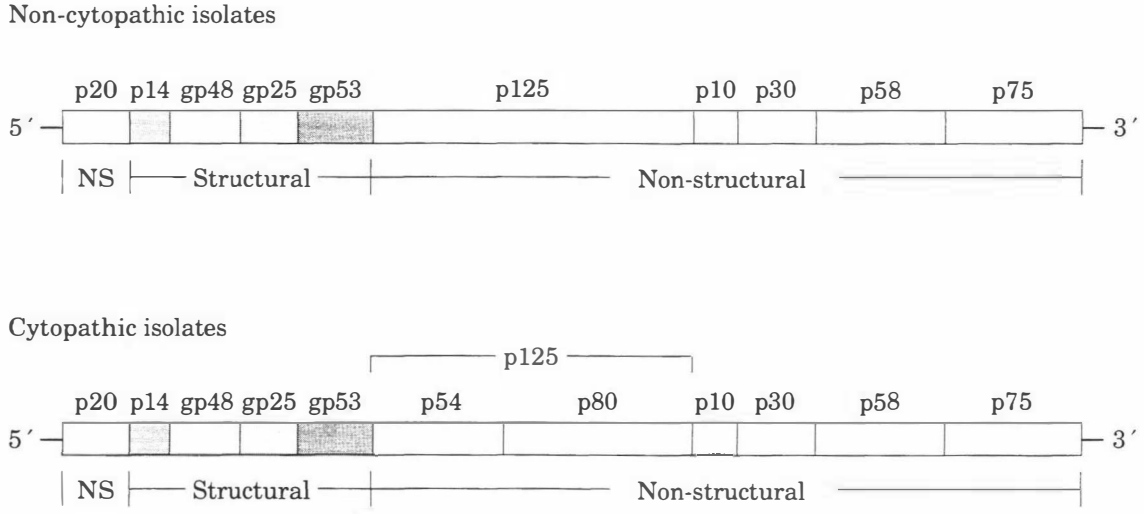


Figure 1.1. Diagramatic representation of final protein products of non-cytopathic (NCP) and cytopathic (CP) bovine pestiviruses. Nettleton and Entrican (1995).

1.3 HISTORY

The following is a summary of significant events in the emergence of bovine pestivirus disease.

1946 - The disease was first recognised in the U.S.A. (Olafson *et al.*, 1946).
Epidemics of acute and often fatal disease occurred. Diarrhoea, with erosive lesions of the digestive tract, was a feature of the disease, and because it was transmissible with material which contained no visible organism, it was termed "Virus Diarrhoea".

1953 - "Mucosal Disease" was first described (Ramsey and Chivers, 1953).
Differences in the degree of severity, chronicity and prevalence made mucosal disease a clearly separate entity from "Virus Diarrhoea" .

1954 - First reported experimental infection (Baker *et al.*, 1954).
Two of 105 calves infected (with an isolate recovered from a sick cow in 1948 in New York State) died, with oral ulcers being the only lesions seen grossly and microscopically.

1957 - A virus causing cytopathic effect in bovine kidney cell cultures was recovered from MD cases (Underdahl *et al.*, 1957).

1961-1963 - Bovine virus diarrhoea (BVD) and mucosal disease (MD) were found to be caused by serologically similar viruses, and clinical, pathological and virological comparisons confirmed that BVD and MD were caused by the same virus (Gillespie *et al.*, 1961; Thomson and Savan, 1963).

1967 - BVD was first confirmed in New Zealand (Jolly *et al.*, 1967)

1968 - The term 'BVD-MD Complex' became official (Jensen *et al.*, 1968).

1978 - First description of an immunotolerant animal, a bull, which was persistently infected throughout its life (Coria and McClurkin, 1978).

1979 - Mucosal disease was confirmed as a late sequel to foetal infection (Roeder and Drew, 1979).

1984-1985 - Mucosal disease was reproduced experimentally (Brownlie *et al.*, 1984; Bolin *et al.*, 1985a).

1987 - First reports of haemorrhagic syndrome in North America due to virulent non-cytopathic BVD viruses (Perdrizet *et al.*, 1987).

1988 - Molecular cloning and nucleotide sequencing of the BVDV genome was carried out (Collett *et al.*, 1988).

1990 - Reclassification of the genus *Pestivirus* from the family *Togaviridae* into the family *Flaviviridae* (Horzinek, 1990).

1993 - Severe outbreak of Type 2 disease in Canada, with the death of 32,000 calves (Carmen *et al.*, 1994, Pellrin *et al.*, 1994).

1.4 SEQUELAE TO INFECTION

The pathogenisis of BVDV infections is reviewed in this section. Experimental infections are described where appropriate, and both virological and pathological aspects are included.

1.4.1 Acute BVDV infections (subclinical ; BVD)

Acute BVDV infection in cattle is generally mild, and often inapparent to the stockman. This subclinical form is the most common sequel to infection. An estimated 70% of cattle seroconvert to BVDV by four years of age in Britain (Harkness *et al.*, 1978) while in New Zealand approximately 60% of adult cattle, whether dairy or beef, have

neutralising antibodies to BVDV (Perez *et al.* 1994; Perez *et al.*, 1995). However, close examination of infected animals will often disclose a rise in temperature, a leukopenia from 3-7 days post-infection (p.i.), and a mild nasal discharge (Brownlie, 1990).

The BVDV isolated from acute infection is non-cytopathic and illustrates that this is the biotype normally circulating within the cattle population (Brownlie, 1990).

With acute infections there is only limited recovery of virus (from blood and nasal secretions) during the first 3-10 days p.i. and by 14 days p.i. virus cannot be isolated. A rise in neutralising antibody starts around this time, and slowly increases to reach a maximum 10-12 weeks post-infection (Howard *et al.*, 1989).[see Figure 1.2] Although virus can only be isolated within a few days after infection, this is followed by an antibody response which persists in the apparent absence of virus. It has been shown that viral antigen is present in the macrophages within the lymphoid tissues of foetuses following experimental infection (Bielefeldt Ohmann, 1983). It appears that non-infectious viral antigen is being continually presented to the immune cells for 10-11 weeks following infection, as there is no evidence that infectious virus remains latent in lymphoid tissues (Brownlie, 1990).

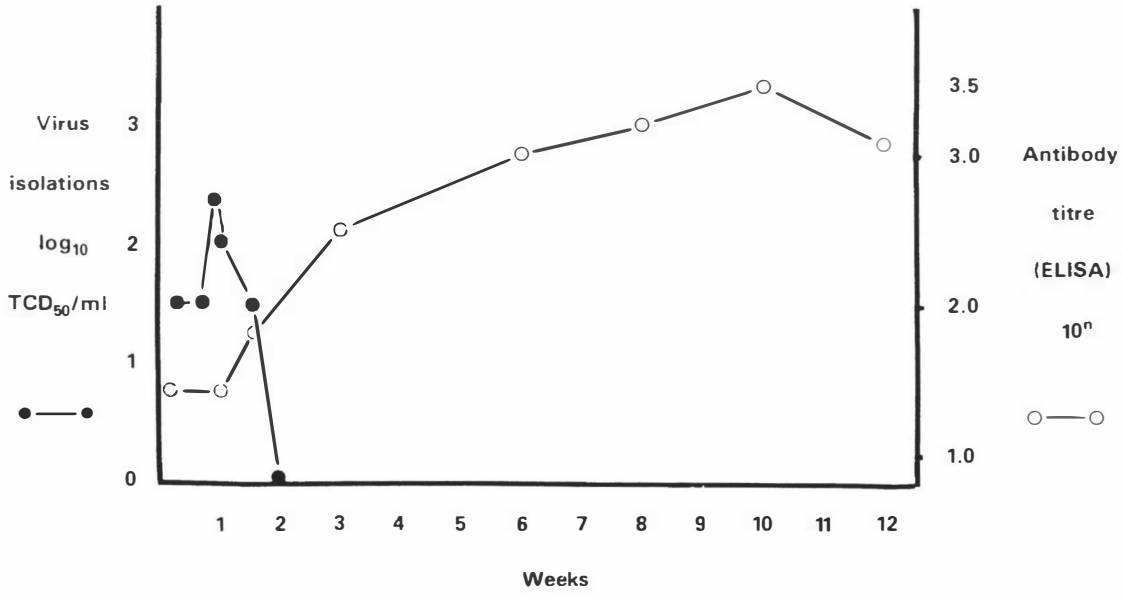


Figure 1.2. Typical virus detection and specific antibody response following acute BVDV infection of calves. Brownlie, 1990.

During acute infection BVDV first replicates in the nasal mucosa, and also reaches high titres in the tonsils, after which it spreads to the regional lymph nodes and then disseminates throughout the body where it reaches high titres in lymphoid tissues (Bruschke *et al.*, 1998). The replication in the nasal mucosa may account for the limited oculonasal discharge and shallow erosions seen in some acute infections (Baker, 1987). In calves, the recovery of virus from nasal secretions appears to be age related, being greatest in young calves (Brownlie, 1990). Systemic spread may occur as cell-free virus in serum, or as virus within cells of the buffy coat fraction, especially lymphocytes and monocytes (Truitt and Schechmeister, 1973). One study investigated both *in vivo* and *in vitro* infection of white blood cells and found that virus was present in peripheral blood mononuclear cells, but at a very low titre compared with tissues such as tonsil, thymus, ileum and epithelium (Bruschke *et al.*, 1998). They concluded that mononuclear cells in the blood were probably not as important as other cells with respect to replication, but that they play a role in the dissemination of virus throughout the body.

During the leucopenic phase, there is a transient decrease in both B and T lymphocytes with recovery to normal levels by 11-17 days p.i. and few clinical signs (Bolin *et al.*, 1985). It would appear that this transient reduction in lymphocytes may not prejudice the ability of the normal young calf to recover from infection. However, a more severe infection can occur in animals older than 6 months (Barker *et al.*, 1993). This form of acute BVD is a common syndrome in rising yearling cattle in their first winter in N.Z. and presents as diarrhoea and ill thrift often in association with other enteric pathogens. This condition has been described by Woods (1994) and the subsequent detrimental effects (eg on mating) are outlined. However, this syndrome has not been reproduced experimentally, so the pathogenesis remains unclear (Brownlie, 1990).

1.4.2 Mixed BVDV infections

A complication of acute infection occurs when another pathogen is involved. It appears that mixed infections are the result of the immunosuppresive effect of the BVDV infection. See section 1.5 for a detailed discussion on this subject.

1.4.3 BVDV infections around mating

Bovine pestivirus infection is associated with significant early reproductive loss in cattle, including fertilization failure, embryonic mortality, and abortion (McGowan and

Kirkland, 1995). The close contact between infected and seronegative cattle at mating time can result in disease transfer by two methods.

A. Venereal infections

This route of infection involves the transfer of infected semen to a susceptible cow or heifer. This is most commonly from a persistently viraemic bull by either natural mating or artificial insemination (Meyling and Jensen, 1988). In addition, semen from immunocompetent bulls undergoing acute BVDV infection may be transiently infected (Whitmore *et al.*, 1978). The testicular tissues are infected, and the semen is often of poor quality (Whitmore *et al.*, 1982). The adverse effect on conception is attributed to fertilisation failure (Grahn *et al.*, 1984). Seronegative cows inseminated with infected semen generally fail to conceive until they develop an immune response to the virus (McClurkin *et al.*, 1979). The risk of venereal infection with BVDV appears to be highest with natural service (Grahn *et al.*, 1984). Following intra uterine infusion of BVDV, recovery of virus was possible for up to two months (Archbald and Zemjanis, 1977). It is possible for an infected cow to infect a bull. An unusual case has been reported recently where a bull at an artificial breeding centre was found to be consistently shedding virus in its semen, despite high antibody titres in the blood (Voges *et al.*, 1998). This is the first published report of an immunocompetent, non-viraemic bull which persistently shed bovine pestivirus in its semen. The blood-testis barrier is cited as the means by which this is possible. One possible explanation for this situation would be that it arose from a venereal infection; from an infected cow back to the bull.

B. Other infections at mating time.

Female cattle can be infected by PI or acutely infected herdmates, especially the bulls.

(a) Infections immediately prior to insemination can cause an ovaritis with resultant failure of ovulation, or delayed ovulation and a subsequent return to service in a normal time interval of about 20 days (McGowan *et al.*, 1993a). Thus, BVDV infection can contribute to the "repeat breeding" syndrome seen in cattle, with the most notable finding being an increase in the number of services per conception (Baker, 1990).

(b) Infections around mating time can also result in embryonic death, with returns to service at a variable time, but always more than 30 days after insemination (McGowan *et al.*, 1993a). Consequently, BVDV infection can increase the number of "late returns" seen after mating.

These two factors have been shown to have a compounding effect in reducing the

overall conception rates in infected cows. In one herd infected with BVDV, the immune cows were shown to have a conception rate of 79%, while only 22% of the infected cattle conceived (Virakul *et al.*, 1988). Experimental studies with susceptible heifers indicate that infections around the time of insemination can significantly increase reproductive losses (McGowan *et al.*, 1993a). They used methods of infection which mimic those that occur in nature. One group of heifers were infected intranasally nine days prior to insemination, to assess the outcome of pestivirus infection during the pro-oestrus and oestrus period, while another group were infected by contact with PI animals four days after insemination, to assess the outcome of pestiviral infection during the early embryonic period, following shedding of the protective zona pellucida. The reproductive performance of these two groups were compared with a control group which did not become infected during pregnancy. Conception rates were determined by serial serum progesterone assays and transrectal ultrasonography, and a final pregnancy test was carried out by manual palpation at 77 days. The results of their experiment are summarised in Table 1.1.

Infection Period	**Infection Method**	**Time of Infection**	**Conception Rate** (20 days)	**Pregnancy Rate** (77 days)
No infection	Nil	Nil	79%	79%
Pro-oestrous /Oestrous	Intranasal	9 days pre-insem.	44%	39%
Embryonic period	Contact with PIs	4 days post.-insem.	60%	33%

Table 1.1. Increased reproductive losses in cattle infected with bovine pestivirus around time of insemination (McGowan *et al.*, 1993a).

The reduced conception rates and very low pregnancy rates in the infected groups were all statistically significant. In the group infected after insemination, the conception rate (at 20 days) was slightly lower than the controls (60% compared with 79%) but then some heifers showed progressive and significant loss of embryos through to day 77 when only one third of the group remained pregnant. In summary, this study has quantified the extent of reproductive failure that can occur in heifers infected with BVDV around mating. A field investigation carried out with beef cattle also confirmed that infection of susceptible females with BVDV around the time of AI could

significantly lower the pregnancy rate (McGowan *et al.*, 1993b). Similar situations of high stock density are often encountered in intensive heifer breeding programmes in the field, when the impact of this virus could be substantial.

A further significant finding in the experiment carried out by McGowan *et al.* (1993a) was that no persistantly viraemic calves were subsequently born, suggesting that the only sequellae to infection around the time of insemination are failure to conceive, embryonic loss, or the birth of a normal non-viraemic calf.

1.4.4 BVDV infection during pregnancy

BVDV rarely infects the foetus of a seropositive cow, where antibodies apparently prevent viraemia or the access of virus through the placentome. The problem of *in utero* infections appears to only occur in the sero-negative dam, where foetal infection can follow from either acute or persistent viraemia (Brownlie, 1990).

The sequel to infection of the seronegative dam depends on the stage of pregnancy (see Figure 1.3) :-

A. Embryonic loss.

This can occur in the first 30-45 days, but the pathogenesis is uncertain, because although the virus is shown to replicate freely in the maternal placenta (Parsonson *et al.*, 1979), contact between maternal epithelium and foetal trophoblast may not be sufficiently intimate for transplacental infection to occur (Whitmore *et al.*, 1978). Embryonic loss could well be a consequence of infections that occur around mating, as described in the previous section.. By 30-45 days of gestation the placenta is fully functional and foetal infection can occur (Kendrick, 1976).

B. Abortion.

Following placentation, the outcome of infection with the non-cytopathic biotype can be death of the foetus, resulting in either abortion or mummification (Kendrick, 1971). Abortion was a feature of the disease when it was first described in New York (Olafson *et al.*, 1946). Foetal death can be a direct result of viral invasion, or may be secondary to damage to the maternal placenta through disruption of its vascular supply of nutrients (Brownlie, 1990). Experimental infections during the first and second trimesters have shown that more than 30% of foetuses are aborted (Brownlie *et al.*, 1986). Recovery of virus from aborted tissues was infrequent in this experiment and this may suggest that placental damage was the most significant factor causing abortion.

The time interval between infection and abortion varies considerably. One experiment using intramuscular injection of BVDV recorded abortions 18-21 days later (Virakul, 1988). In the field however, abortions can take place several months after infection (Brownlie, 1990). Mummified foetuses may remain *in situ*, even beyond the expected calving date.

Experimental infection of cattle during the first trimester of pregnancy with the CP biotype does not cause abortions, and there is doubt whether this biotype can even establish in the early foetus (Brownlie *et al.*, 1989).

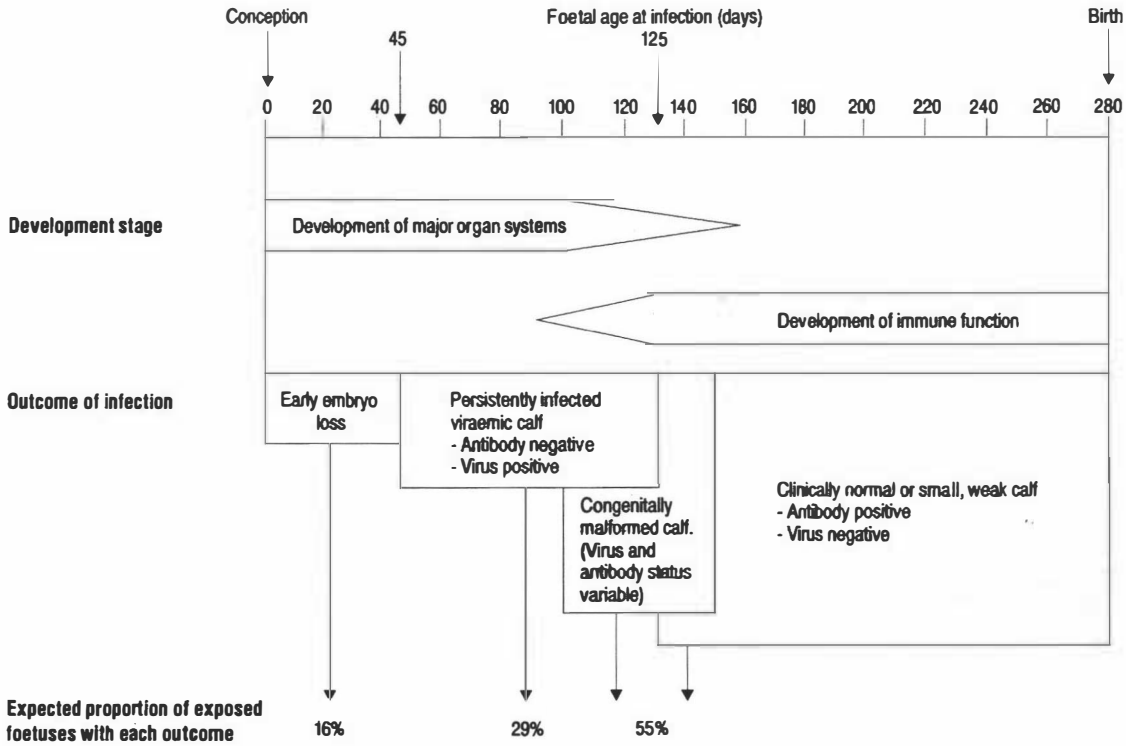


Figure 1.3. The consequences of bovine foetal infection with BVDV. Harkness and van der Lugt, 1994.

N.B. Abortion can occur at any stage following placentation (i.e. from 45 days on).

The pathology of the aborted foetus after experimental infection is described by Jubb and Huxtable (1993). Gross lesions are the result of a necrotising inflammatory reaction which can involve a variety of tissues. The reactive changes are dominated by mononuclear cells, predominantly macrophages, which infiltrate hepatic portal areas, myocardium, spleen, and lymph nodes. This is seen grossly as enlargement, nodularity and mottling of the liver, myocardial pallor (due to myocarditis and degeneration of the heart muscle), and enlargement of spleen and lymph nodes. In addition, growth-arrest lines can be seen in long bones. This suggests that the foetus undergoes one or more intrauterine crises before death ensues. Affected foetuses may show partial alopecia, which spares the tail, lower limbs and head, these being points of inital hair growth during foetal development. Microscopically there is hypoplasia of hair follicles and cystic distension of adnexal glands.

C. Teratogenesis.

Infections that occur in the foetus during organogenesis (up to day 150-170) can result in teratogenesis. BVDV has a preference for mitotically active cells, particularly those of the central nervous system (CNS) and lymphoid tissues (Done *et al.*, 1980). A further consistent finding is localisation in the vascular endothelium, resulting in vasculitis and degeneration of target tissues (Van Oirschot, 1983). The resulting pathological defects have been outlined (Baker, 1990; Jubb and Huxtable, 1993).

Cerebellar hypoplasia is the most characteristic defect seen. The evolution of the cerebellar changes has been studied experimentally (Ward *et al.*, 1969). Within two weeks of infection, acute lesions were seen and changes continued to take place up to the sixth week. By ten weeks after maternal infection, inflammatory changes were no longer evident in the brain.

Other CNS defects which may be a consequence of foetal infection are porencephaly, hydranencephaly, microencephaly, hydrocephalus, cystic septum pellucidum and dysmyelination.

Ocular defects commonly accompany cerebellar defects, and the anomalies seen are retinal atrophy and dysplasia, cataract, optic neuritis and atrophy, micropthalmia, and persistent pupillary membrane.

Immune system defects - especially thymic aplasia.

Integument defects - hypotrichosis and alopecia.

Musculoskeletal defects - brachygnathism, growth retardation and arthrogryposis.

Respiratory system - pulmonary hypoplasia.

Once organogenisis is complete (by about 170 days), subsequent infections are unlikely to result in malformation of the foetus (Jubb and Huxtable, 1993).

D. Growth retardation

The ability of the bovine foetus to mount an immune response develops after about 90 days of gestation, and nearly all foetuses are capable of producing antibody to BVDV by 125 days (Harkness and van der Lugt, 1994). An infection after this time can result in a successful immune response where the calf is born clinically normal, or can result in growth retardation with the birth of a small, weak calf (Duffell and Harkness, 1985). Both will be negative for virus, and with an antibody titre to BVDV. (Harkness and van der Lugt, 1994). Growth retardation is manifested as stunting, lower birth weights, and lower organ weights (Duffell and Harkness, 1985). Growth retardation can be diagnosed radiographically by a shorter than normal tibial diaphyseal length or by the presence of metaphyseal lines (Constable *et al.*, 1993). It is suggested that the teratogenic effect of the virus on the immune system (eg. thymic atrophy) could contribute to this syndrome (Jubb and Huxtable, 1993) as there is lowered resistance to pathogens such as enteric and respiratory organisms. This could also be a direct effect of the virus, as it has been well established that acute BVDV infections can result in immune suppression of the host (Potgieter, 1988).

E. Persistent viraemia

McClurkin *et al.* (1984) describe another outcome of foetal infection during the first trimester, after development of the foetal membranes, but before the immune system is functional (about 45-125 days). Foetal infection at this time can result in the establishment of a viraemia that persists for life. At this time the foetal immune system is not developed sufficiently to recognise BVDV as foreign. Once the immune system does start to function (after 100-125 days), the virus is accepted as a "self" tissue, and immunotolerance results. The virus is thus able to persist in the blood and tissues for the lifetime of the animal and there is no antibody produced against it. In all the recorded field and experimental data there is no evidence for persistence with the cytopathic biotype (Brownlie *et al.*, 1989). That is, only the non-cytopathic biotype is involved in the production of persistently infected (PI) calves.

The clinical appearance of PI animals can range from normal to grossly abnormal. In

one study of twelve PI calves over a two year period, one calf remained healthy, two became stunted, one was found to be infertile, three developed pneumonia and three developed mucosal disease (Barber *et al.*, 1985). Half of these calves died during this period, one inexplicably. The reason why some PI animals are more damaged than others is not fully understood, but it may be related to the timing of infection. If BVDV infection occurs when the lymphoid organs are starting to develop, then this could result in permanent damage to the immune system and subsequent lowered resistance to infections. As indicated in the above study, many PI calves become stunted in growth and in the field these are seen as "runts" in the mob. Despite the high prevalence of BVDV in cattle populations, only a small percentage (1-2%) are persistently infected with this virus (Littlejohns and Horner, 1990). The implication of this is further discussed in section 1.7.

The pathogenesis for the abnormal PI calf is related to the viral tropism for CNS, lymphoid and epithelial cells, as already discussed.

- Within the CNS, the predilection sites for viral persistence are the cerebral cortex and the hippocampus (Fernandez *et al.*, 1989). Lesions here are often more severe when the foetus is infected late in the expected period (second trimester) and account for the depression and incoordination seen in some newborn PI calves. Often these calves fail to survive and brain lesions such as cerebellar hypoplasia can be seen at necropsy (Done *et al.*, 1980).

- Lesions within lymphoid tissues are not marked, although reduction in size may be evident, especially of the thymus (Done *et al.*, 1980). In contrast to MD, the Peyer's patches in the small intestine show little change (Brownlie *et al.*, 1984). However, there are cellular changes which may account for the immunosuppression seen in persistently viraemic animals. There is a reduction in the recirculating B cells (Muscoplat *et al.*, 1973) and T cells (Reggiardo and Kaeberle, 1981). It has been estimated that 4.4% of blood leucocytes, 5.4% of T cells and 2.1% of B cells are infected with virus in PI animals (Bolin *et al.*, 1987).

- Several epithelial tissues sustain BVDV replication in the PI animal. BVDV antigen can be demonstrated within the keratinocytes of the tongue, skin and labia (Bielefeldt Ohmann, 1983).

Persistent infection with BVDV means the animal is antibody negative despite widespread viral infection. This contrasts with the persistence seen with other viral infections, such as Infectious Bovine Rhinotracheitis (IBR) and Enzootic Bovine Leucosis (EBL), where the carrier animal is always antibody positive (Deregt and Loewen, 1995). In addition, there is no evidence that the state of latency, as seen with IBR virus, occurs with BVDV (Brownlie, 1990).

1.4.5 Mucosal Disease

A. Pathogenisis.

Mucosal disease (MD) is a sporadic disease in cattle, generally occurring between the age of six months and two years (Baker, 1990). Clinical signs are severe, morbidity is low, but the death rate high, approaching 100%. Usually less than 5% of the herd is affected, but occasionally epizootics may involve up to 25% of the animals in a herd.

Although first reported in 1953, the virus isolated from this condition did not reproduce the fatal MD following inoculation into cattle (Ramsey and Chivers, 1953). It was not until some 30 years later that MD was reproduced experimentally (Brownlie *et al.*, 1984; Bolin *et al.*, 1985). This followed the discovery that only persistently viraemic animals succumb to MD (Liess *et al.*, 1974), and that both biotypes of the virus are always present (Brownlie *et al.*, 1984). The pathogenesis was then clarified experimentally. A calf persistently viraemic with NCP virus was superinfected with CP virus and this resulted in the rapid development of fatal MD (Brownlie *et al.*, 1984; Bolin *et al.*, 1985). Subsequently, it was recognised that it was the "homology" between the persisting and superinfecting biotypes that determined whether the disease course was short or long (Brownlie *et al.*, 1987). If the biotypes were "homologous" (antigenically similar to one another), then death ensued in 2-3 weeks. This would certainly be the case if the CP virus arose by mutation from the NCP one. On the other hand, if the CP virus is different antigenically, an immune response can be mounted, which would differ according to the degree of "heterology". Experimental studies using partially "heterologous" CP virus resulted in chronic disease for several months before euthanasia became necessary (Brownlie *et al.*, 1986). Cattle with chronic MD may survive up to 18-24 months and ultimately die from severe debilitation (Baker, 1990).

Based on the distribution of viral antigen within tissues and the lesions seen, the following pathway of infection has been proposed for mucosal disease (Barker *et al.*,

1993). Circulating precursor cells of macrophages (monocytes) take up the virus from the mucosal cells in the respiratory tract and tonsils, and transport it intracellularly to the lymphoid tissues and to the subepithelial connective tissues of the dermis and the gastrointestinal tract, whence it spreads to overlying epithelial cells. Specific immunotolerance in the PI animal may allow a closely related CP biotype to freely infect, replicate, and destroy cells. Barker *et al.* (1993) suggest that the chronic effects of the NCP virus on the cells may well enhance the replication of the superinfecting CP virus.

B. Clinical signs and pathology.

1. Acute mucosal disease

Clinical signs can be dramatic. Acute fulminating mucosal disease has been described by Barker *et al.* (1993) as closely resembling rinderpest. The distribution of lesions relates to the affinity of BVDV for epithelial cells in the gastro-intestinal tract, integument, and respiratory tract, and endothelial cells of blood vessels, especially in the intestines and lymphoid tissue (Baker, 1990). The following summary has been drawn from a review of 14 papers by Baker (1990) :-

Cardinal signs can be dramatically altered (pyrexia, polypnea, tachycardia), as can the general state of the animal (depression, weakness, anorexia). Erosive lesions may be observed in the oral cavity (lips, gingivae, tongue, dental pad, commisures of mouth and posterior part of hard palate). Lesions may coalesce to form large areas that slough. The external nares and nasal cavity can also develop erosive lesions. Salivation often accompanies the oral lesions which are found in 75-80% of the cases. Mucopurulent nasal discharge is often observed, while lacrimation and corneal oedema sometimes occur. Lameness may be seen due to skin lesions (erosions and necrosis) in the interdigital cleft, while coronitis and even laminitis also occur. Diarrhoea develops 2-3 days after the onset of clinical signs, and the profuse watery faeces may contain variable amounts of fresh or clotted blood. Ruminations are decreased, and mild to moderate bloat may develop.

Clinical pathology may reveal a severe leukopenia in the early stages of disease. Neutropenia without a left shift, lymphopenia and thrombocytopenia may develop. Secondary bacterial infections are common.

Death may occur in the acute phase of the disease, but more commonly takes place 3-10 days after the onset of clinical signs. Although dehydration with acidosis develops as the disease progresses and may sometimes be the cause of death, it must be noted that

death may occur prior to the onset of diarrhoea in peracute cases. The actual cause of death in mucosal disease remains obscure.

Post-mortem findings include erosions in the upper alimentary tract (oro-pharynx, oesophagus, rumen pillars, omasum). The blunting of oral and rumen papillae may also be observed. Oedema and haemorrhage of the pyloric portion of the abomasum can occur. Catarrhal enteritis is seen, with intestinal contents being dark and watery. The enteritis may progress to include haemorrhage, erosions and ulcers of the mucosa. Swollen and haemorrhagic Peyer's patches may be seen through the serosa of the ileum. Congestion of the mucosa of the large intestine often presents as a 'striping' pattern.

Histologic examination, as briefly summarised by Baker (1990), reveals varying degrees of necrosis in the germinal centres of lymph nodes and spleen. Oedema and inflammatory cell infiltration is seen in varying degrees throughout the gastro-intestinal tract. An extensive review of the histopathology of mucosal disease has been made by Barker *et al.*, 1993, and this is covered in Chapter 3.

2. Chronic mucosal disease

A small proportion of cattle that develop mucosal disease do not die in the expected time frame and become chronically affected (Baker, 1990). This is characterised by inappetence, weight loss, progressive emaciation and an overall unthrifty appearance. Diarrhoea may be continual or intermittent. Chronic bloat may be observed. Nasal discharge and persistent ocular discharge are frequently found. Areas of alopecia and hyperkeratisation can develop, especially in the neck. Chronic erosive lesions can be found in the mouth and on the skin. The areas of skin most often affected include the perineum, prepuce, vulva and skin/horn junctions such as the dew claws, interdigital cleft and heels. The failure of skin lesions to heal is an important finding in chronic mucosal disease. Chronic lameness may occur due to laminitis, interdigital necrosis or hoof deformities. Secondary bacterial infections are common. Chronic pancytopenia can occur (anaemia, leukopenia, neutropenia and lymphopenia). Cases may survive up to 18 months of age, and death is from severe debility.

3. Treatment of MD.

Treatment is not warranted, and after the diagnosis is confirmed, the animal is best removed from the herd and further testing of the mob carried out to determine the presence of any persistently infected cattle, which should also be removed (Baker, 1990).

1.4.6 Type 2 BVDV infection

As discussed previously, BVDV is now segregated into two genotypes - Types I and 2. A review by Horner (1996) describes genetic studies which have shown that there is about 75% sequence homology between type I and type 2 viruses, whereas within types it is 90-96%. In addition, although there is some cross-neutralisation, hyperimmune type 1 sera only has titres of 50-100 against type 2 viruses, compared to 800-12,800 against type 1. It has been reported that type 2 BVDV has been isolated from PI calves born to dams vaccinated against BVDV using live vaccine (Ridpath *et al.*, 1994). It has been suggested that cross protection may not have been sufficient to prevent transplacental infection (Baker, 1995). Type 2 viruses are noncytopathic, and cause severe disease.

A. Haemorrhagic syndrome

Thrombocytopenia was reported in association with acute BVDV infection in America in the 1980's (Perdrizet *et al.*, 1987) and experimentally induced thrombocytopenia was also reported (Corapi *et al.*, 1989). In this study two BVDV isolates caused a profound decrease in platelets (counts of less than 5000 were seen in 12 of 13 calves from 3 to 11 days post infection) and multiple haemorrhages occurred throughout the body in 11 of 13 calves. Thrombocytopenia with haemorrhages had been recognised in adult cattle (Rebhun *et al.*, 1989) and veal calves (Copari *et al.*, 1990) in association with non-cytopathic isolates of BVDV (Rebhun *et al.*, 1989). It was later confirmed that this haemorrhagic syndrome was due to infection with type 2 BVDV (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994).

Haemorrhagic syndrome is characterised by severe thrombocytopenia, bloody diarrhoea, epistaxis, haemorrhages on mucous membranes, and bleeding from injection sites (Rebhun *et al.*, 1989). The main sites of haemorrhage are the subcutis, and most serosal surfaces, especially the small intestine and mesentery, where massive haemorrhage occurs (Barker *et al.*, 1993). In calves there may be haemorrhage into the anterior chamber of the eye, and scleral haemorrhages may also be present (Baker, 1995). Other signs are fever, leukopenia, anaemia, and the thrombocytopenia may persist for 6 weeks.

Thrombocytopenia is the basis for this haemorrhagic syndrome, and this has been reproduced experimentally (Copari *et al.*, 1989; Rebhun *et al.*, 1989, Copari *et al.*, 1990). BVDV-specific antigen was detected in megakaryocytes using immunohistochemical staining (Marshall *et al.*, 1996) and it was proposed that the pathogenesis of the thrombocytopenia may be related to megakaryocyte degeneration

and abnormalities in the ultrastructure of platelets, as described for hog cholera virus (Weiss et al.,1973).

Haemorrhagic disease has been reproduced experimentally (Bolin *et al.*, 1992; Odeon *et al.*, 1999). One study resulted in low morbidity and mortality rates (Bolin *et al.*, 1992). Passively derived antibodies to BVDV protected calves from experimental challenge. In another study, the concurrent use of steroid injections (to mimic environmental stress) along with a NCP field isolate of BVDV genotype 2 resulted in severe and fatal disease (Odeon *et al.*, 1999). There was haemorrhagic diarrhoea and multifocal haemorrhages in the gastrointestinal tract. Some of the lesions recorded were typical of mucosal disease (lymphoid, intestinal and respiratory lesions) while others were not present (oral and oesophageal erosions). However only three calves were used in this experiment, with no control animals without steroids. Consequently, the significance of the steroid effect is difficult to gauge.

B.Peracute syndrome

In North America in 1993 very severe outbreaks of acute BVDV infection occurred involving 850 herds, with an overall mortality of 32,000 calves (Horner, 1996). These involved both Canadian and American states that border the Great Lakes region, with reports coming from Ontario (Carmen *et al.*, 1994), Quebec (Pellerin *et al.*, 1994) and Pennsylvania (Drake *et al.*, 1994). It was estimated that 25% of veal calves died of BVDV infections in Quebec. In Ontario, the clinical signs associated with this severe form of pestivirus infection included pyrexia, pneumonia and diarrhoea in all age groups. Oral ulcers and abortion were common in older cattle. Mortality rate was variable between herds, reaching 20% in some. Outbreaks were reported to be associated with recent introduction of cattle to the herd. The majority of isolates were NCP and were typed as BVDV, type 2 (Baker, 1995).

Pyrexia with sudden death in association with type 2 BVDV has been more widely reported in America since then. For example, a case study in a herd of Simmental cattle in Virginia, USA, was reported by Swecker *et al.* (1997). Type 2 non-cytopathic BVDV infection was diagnosed based on virus isolation, serology and PCR testing. Severe disease occurred in both cows and calves. In calves the disease was marked by profuse salivation, liquid faeces, dilated pupils, fever, depression, anorexia and death within 72 hours. Cows consistently exhibited diarrhoea, with a clinical course of 1 to 17 days. There was 39% mortality in the calves and 27% in the cows. The only animal introduced to the farm was a bull that was used to mate the cows, but this was not

tested. There was a history of use of inactivated BVDV vaccine 2-3 years before the disease outbreak.

Similarly severe and often fatal forms of BVDV infection were reported in dairy herds in Britain in 1992 and 1993 (Hibberd and Turkington, 1993; David *et al.*, 1994). Severe disease in adult cattle in three dairy herds in Britain associated with type 2 non-cytopathic BVDV has been documented (David *et al.* 1994). In one herd there was 40% morbidity and 10% mortality among adult cows. Pyrexia, watery diarrhoea and agalactia were consistent clinical signs. The source of infection was unknown on two farms, and in the third was traced to purchase of a PI heifer.

To date there has been no evidence of type 2 BVD infection in New Zealand cattle (Vilcek *et al.*, 1998). However, in this study, testing of a biological product of American origin resulted in the isolation of a BVDV with a type 2 genotype. Another study indicated that the type 1 group contains BVD viruses that are used in vaccine production, diagnostic tests and research studies, whereas the type 2 group represents isolates from foetal calf sera, PI calves born from dams vaccinated against BVDV (type1) and cattle dying from peracute syndrome or haemorrhagic syndrome (Ridpath *et al.*, 1984). It is possible that the type 2 virus is a mutant NCP form which may need to be included in future vaccines for control of the disease (see section 1.8).

1.4.7 Summary of clinical syndromes.

The clinical aspects of BVDV infections were reviewed by Baker (1990, 1995) and the following table has been drawn from these papers, as well as summarisng data from the previous section (see Table 1.2).

"Illthrift" is a feature of three of the syndromes, namely bovine virus diarrhoea, persistent viraemia, and chronic mucosal disease. These can sometimes be differentiated on clinical signs or on the timing of the signs (Baker, 1990) However, laboratory testing is often needed to make a differential diagnosis (see section 1.6).

Suppression of the immune system is a feature of many of the clinical syndromes, including acute or chronic infections and type 1 or 2 strains of virus. Because of the importance of immunosuppression, the following section (1.5) has been devoted to this aspect of BVDV infection.

INFECTION TYPE	CLINICAL MANIFESTATION	AGE and COMMENT
Subclinical infection	No clinical signs seen	Any age- occurs in previously seronegative animals
Bovine virus diarrhoea	Diarrhoea, illthrift & immune suppression	6-12 months-especially winter
Mixed infections	Respiratory, enteric, immunosuppression	Young cattle especially
Venereal infections	Fertilisation failure and repeat breeding	Breeding age females
Other infections at mating	Repeat breeding and late returns	Breeding age females
Congenital infections		*Time of gestation (days)*
	Embryonic loss and late returns	Prior to placentation ~(0-45)
	Abortion, mummification, stillbirth	Following placentation (at ~45)
	Calves born persistently infected	Pre immune function ~(45-125)
	Congenital defects	Organogenisis (up to ~170)
	Weak & undersized calves	Immune function (after ~125)
	Calves born normal & seropositive to BVDV	Immune function (after ~125)
Persistent viraemia	"Normal" or "Runts"- illthrift and immunosuppressed	50% death rate in first year
Mucosal disease	Severe, sporadic & fatal disease	6 months to 2 years of age
	1. Acute mucosal disease	Death in 2-3 weeks
	2. Chronic mucosal disease	Death within 18 months
Type 2 infections	1. Haemorrhagic disease	Thrombocytopenia
	2. Peracute disease	Sudden death is common

Table 1.2 Summary of the clinical manifestations of bovine pestivirus disease. After Baker, 1990 & 1995.

1.5 IMMUNOSUPPRESSION

BVDV may be a pivotal component in multiple-cause infectious diseases, and laboratory evidence indicates that BVDV causes a profound and broad spectrum deficit in the immune response in cattle (Potgieter, 1997).

1.5.1 Pathogenesis of immunosuppression

The immunosuppressive effect of BVDV is based on the fact that the virus has a strong affinity for immunocompetent cells, which may be destroyed, or survive in a functionally impaired state (Potgieter, 1995). At the cellular level, BVDV depresses the production of immunoglobulin and interferon, reduces the response of lymphocytes to mitogens and impairs monocyte chemotaxis (Harkness and ver der Lugt, 1994). This depression of cell-mediated immunity is accompanied by a lack of humoral antibody response (Barker *et al.*, 1993). The virus replicates in lymphocytes and macrophages in culture, while *in vivo* there is a decrease in the absolute numbers of circulating B and T lymphocytes (Bolin *et al.*, 1985). Depression of polymorphonuclear function occurs through a decrease in total numbers (Roth and Kaeberle, 1983).

The measurable effects of immunosuppression include leukopenia, which occurs in most cattle infected with BVDV, and lymphoid depletion, a frequent post-mortem finding.

One experiment carried out by Reggiardo and Kaeberle (1981) indicated that pestivirus infection in calves promoted the dissemination of endogenous bacteria, by way of a transient bacteraemia. Leukopenia, lymphocyte suppression and bacteraemia coincided temporarily, indicating that the BVD virus impairs the normal blood bacterial clearance mechanisms.

Modified-live BVDV vaccines have been found to cause immunosuppression (Roth and Kaeberle, 1983).

1.5.2 Immunosuppression and respiratory disease

Experimentally, severe fibrinopurulent bronchopneumonia and pleuritis was produced in calves if the pestivirus was introduced directly into the bronchi, followed by *Pasteurella haemolytica* five days later (Potgieter, 1997). There was wide dissemination of *P. haemolytica* within the respiratory tract. With endobronchial inoculation of control

calves with *P. haemolytica* alone, a much less severe localised reaction occurred, with lesions confined to inoculation sites only. In addition bacteria could only be cultured from these inoculation sites. Calves inoculated with BVDV alone, only developed a mild interstitial pneumonia. However, numerous bacteria were recovered from the lower respiratory tract of some of these calves. It was concluded that the BVDV had enhanced the disease caused by *P. haemolytica* both by impairing bacterial clearance from the respiratory tract, and promoting dissemination of bacteria within the lower respiratory tract. This may occur by impairment of the ability of the mucosal epithelium immediately above the peribronchial lymphoid tissue to rapidly internalise the bacteria. Alternatively, or concurrently, infection of alveolar macrophages with BVDV may impair their ability to kill bacteria. Recent *in vitro* studies have shown that with viral infections, including BVDV, fibrin deposition in the pulmonary alveoli prior to the establishment of a secondary Gram-negative bacterial infection, is a consequence of increased output of procoagulants from the alveolar macrophage (Olchowy *et al.*, 1997). If this reaction is similar *in vivo*, then the excess fibrin formation resulting from viral infection of alveolar macrophages would produce an environment favourable to bacterial colonisation.

Concurrent infections of BVDV and IBRV in calves can result in severe clinical disease affecting the respiratory system, alimentary tract and ocular tissues (Greig *et al.*, 1981). In many of these calves, apparent opportunistic infections with *P. haemolytica* further enhanced the severity of respiratory tract lesions. Experimental infections showed that BVDV infection impaired the ability of calves to clear IBRV from the lungs, as well as allowing IBRV to spread outside the respiratory system (Potgieter *et al.*, 1984).

Experimental infections of calves with either BVDV or bovine respiratory syncytial virus (BRSV) alone resulted in mild clinical signs of the upper respiratory tract, whereas with dual infections there was dyspnoea and lower respiratory tract involvement with significant lung consolidation (Kelling *et al.*, 1995).
Serological testing in eight herds in New Zealand which were experiencing respiratory disease revealed evidence of BVDV along with IBRV and BRSV in one herd; IBRV and BRSV in three herds; IBRV alone in one herd, Parainfluenza 3 (PI_3) in one herd, and failed to find evidence of any of these viruses in the remaining two herds (Motha *et al.*, 1997). Serological evidence for BRSV has only recently been reported in N.Z., with

a prevalence of antibodies in dairy stock of 75.6% (Motha and Hansen, 1997).

1.5.3 Persistent infection and immune status

Although a proportion of persistently infected animals remain healthy, others deteriorate, lose condition and often develop pneumonia (Potgieter, 1997). The difference between these 'healthy' and 'unhealthy' PI animals is not fully understood. The ability of persistently viraemic calves to respond to antigens *other than* BVDV has been assessed (Houe and Heron, 1993). Healthy but PI calves showed similar immune responses to the control calves; antibody responses to tetanus immunisation, skin reactivity after immunisation against Johne's disease and skin sensitisation with dinitroclorobenzene. A reduced immune response had been expected in these persistently infected calves. Unfortunately, no unhealthy PI calves were included in this study, as this group would be more likely to be immunocompromised. Theoretically, infection of the foetus with BVDV at the time when its immune system is starting to develop (around 90-100 days) could result in damage to lymphoid tissues, resulting in a reduced immunocompetence. This proposal may also explain why some PI calves do have a low antibody titre to BVDV at birth, as they have been able to mount some immune response (Penny *et al.* 1996).

Further support for immune suppression of PI animals is given by the report that some PI cattle have significantly reduced levels of IgG, as do animals with MD (Barker *et al.*, 1993).

1.5.4 Immunosuppression and other pathogens

Intercurrent BVDV infections seem to enhance the virulence of other pathogens, or change the nature of the disease caused by such pathogens (Potgieter, 1997). For example, it may prolong the disease caused by other organisms, or increase the severity, as described with pasteurellosis. Bovine pestivirus may act as a predisposing factor in many cattle infections, but since its presence is not always clinically apparent, its role is often unrecognised. There is increasing evidence that the bovine pestivirus may predispose cattle to a number of other pathogens.

A. Enteric pathogens.

Concurrent *Salmonella* Typhimurium and bovine pestivirus infection in a group of pregnant dairy heifers presented as a very severe form of acute BVD (Penny *et al.*, 1996). Calves experimentally infected with both BVDV and *S.* Typhimurium showed

more severe clinical signs, and excreted salmonellae in faeces for longer than calves infected with salmonella alone (Wray and Roeder, 1987). This suggests a synergistic effect between these two enteric pathogens.

Escherichia coli infections may be exacerbated by the bovine pestivirus (Potgieter, 1997) and mixed infections with other enteric viruses such as coronavirus and rotavirus have been shown experimentally to result in protracted enteritis (Brownlie, 1990)

The effect of the bovine pestivirus on enteric infections probably relates to its predilection for the Peyers patches in lower small intestine, as well as its affinity for epithelial cells, with damage seen mainly in the crypts of Lieberkhun. This damage to epithelial cells may promote the establishment of other surface pathogens, which can then further invade the intestinal wall due to a compromised local immune system.

B. Uterine pathogens.

It has been proposed that BVDV promotes placental colonisation by bacteria, leading to metritis (Reggiardo and Kaeberle, 1981).

A study of protozoal abortions due to *Neospora caninum* indicates that BVDV is also commonly present (personal communication, N.Williamson, 1999). During the epidemic of acute BVDV infection in Ontario in 1993, there was an increase in the number of diagnosed abortions due to Neospora species (Alves *et al.*, 1996a; 1966b). The immunosuppressive effect of BVDV could trigger a latent protozoal infection.

C. Other infectious diseases that BVDV may exacerbate are; actinomycosis, papular stomatitis, acute helminthiasis and mastitis (Ames, 1986; Bohac and Yates, 1980).

1.6 DIAGNOSIS OF BVDV INFECTION

For diagnosis in live animals, the preferred material for detection of virus or viral components and antibodies is blood. Available tests and their application have been reviewed (Houe, 1996; Horner, 1996). With dead animals a range of tissues are collected (Nettleton and Entrican, 1993).

1.6.1 Detection of antibodies

A. Serum neutralisation test (SNT).

This has been widely used for many years and detects antibody to gp53, which is an envelope glycoprotein associated with virus neutralisation (Horner, 1996). The SNT

takes at least four days to perform, uses cell cultures, cannot be used to test toxic or contaminated serum, and is more expensive than the ELISA test (Horner, 1996). Because neutralisation tests can vary between cell cultures, paired "acute" and "convalescent" samples are best tested together to demonstrate a rise in antibody titre (Houe, 1996). The method used to perform the SNT is outlined in Chapter 2.

B. Enzyme-linked immuno-absorbance assay (ELISA).

A number of different types of antibody-detecting ELISAs have been described that show good correlation with the SNT (Howard *et al.*, 1985; Chu *et al.*, 1991). Horner (1996) describes the ELISA test developed at the Central Animal Health Laboratory (CAHL) in N.Z. as showing good correlation to the SNT, with a sensitivity of 95.2%, and a specificity of 99.9%. Titres are not comparable to the SNT because the ELISA detects antibody to the non-structural p125/80 protein of the BVDV. Since this is only produced while the virus is replicating, it will not detect antibody to inactivated vaccine. One commercial assay is available which detects gp53 antibody, and this can be used to measure antibody responses to vaccination. The ELISA assay is better suited to screening large numbers of samples, and is less expensive than the SNT, so is the main test used in NZ (Horner, 1996).

An indirect ELISA has been developed for use on bulk tank milk samples (Niskanen *et al.*, 1991). This has been used as a tool in the diagnosis and prophylaxis of BVDV infections in Scandinavia (Niskanen, 1993).

1.6.2 Detection of virus or viral components.

A. Virus isolation from serum or tissue.

This detects infectious virus and requires cell culture [e.g. bovine testicle, kidney, foetal lung or nasal turbinate cells]. After 3-5 days incubation, non-cytopathic viral growth is detected by immunolabelling. Immunofluorescent techniques have now been superceded by immunoperoxidase (IP) staining (Meyling, 1984). Virus isolation followed by IP is a very sensitive test, but is more expensive than the ELISA test and takes longer to perform; four days for a one pass test, and 11 days for a two pass test (Horner, 1996).

B. Antigen ELISA, using blood (or tissue).

A number of different BVDV antigen ELISA's are available (Fenton *et al.*, 1991, Brock, 1995). Some of these use monoclonal antibodies, which have been useful in revealing the antigenic diversity among BVDV isolates, as well as in identifying

pestivirus group (BVDV, BDV or SFV).

An ELISA antigen test has been developed for routine detection of BVDV in N.Z., where buffy coat is the preferred sample (Horner, 1996). It is not sensitive enough to detect antigen in serum, but is a robust and cheap test, which takes two days to run.

C. Polymerase Chain Reaction (PCR).

Nucleic acid detection techniques using nucleic acid hybridisation probes and PCR have been developed for BVDV and used diagnostically (Brock, 1995). Specific nucleotide sequences of the viral RNA are measured, and the sensitivity and specificity of this method is dependent on the region of gene selected. However it is the most sensitive test for virus and unlike virus isolation there is no interference by neutralizing antibodies. It is the most expensive test, takes two days to run, and any sample can be used (Horner, 1996). Because of the very high sensitivity, great care must be taken to prevent cross-contamination of samples in the laboratory.

D. Immunohistochemistry.

BVDV has been detected in visceral organs by immunohistochemical means (Bielefeldt Ohmann 1982; Bielefeldt Ohmann, 1988a; Bielefeldt Ohmann, 1988b; Wilhelmsen *et al.*, 1991, Marshall *et al.*, 1996). It has been demonstrated that BVDV is present in skin samples in PI cattle (Bielefeldt Ohmann, 1988a; Bielefeldt Ohmann, 1988b). An immunohistochemical assay has been described that is a rapid and reliable method for the diagnosis of BVDV infection in PI animals (Thur *et al.*, 1996).

1.6.3 Application of tests to clinical cases.

A. Acute infection

The viraemia in cattle acutely infected with BVDV lasts from 2 up to about 15 days, so antigen detection can be limited. Paired serum samples three weeks apart will demonstrate seroconversion or rise in antibody titre (Houe, 1996). Additional serum samples from other herd mates should always be included to increase the detection of acute infection in the herd. Note that since the level of subclinical infection in NZ is in the order of 60%, it is clearly not possible to interpret a single serological test (Horner, 1996). The testing of paired samples is helpful in the diagnosis of "repeat breeding" due to BVDV (Houe, 1996). Diagnosis of abortion due to BVDV is difficult because of the variable time interval between acute infection and expulsion of the foetus. A rise in antibody titre can sometimes be demonstrated, but the antibody level may already be high at the time of abortion. The aborted foetus can be examined for the presence of

virus and antibody, and if either are present this will prove intrauterine infection with BVDV (Houe, 1996). The distinctive histopathology of lesions in the foetus that aborts due to BVDV infections has been previously described (section 1.4.5.).

B. Maternal antibody and foetal infection

Colostral antibody can last in the calf up to eight months of age and may interfere with tests for virus or antigen, especially the IP test (Horner, 1996).

Diagnosis of BVDV infection among neonatal calves therefore requires a blood sample collected before uptake of colostrum, to be tested for both virus (or viral antigen) and antibody so that acute infection and persistent infection can be differentiated (Houe, 1996). Final proof of persistent infection will require a second sample, which may have to wait until colostral antibody has disappeared.

Neonatal calves that are antibody positive at birth may indicate that *in utero* BVDV infection is the cause of any observed congenital defects, growth retardation or lack of vigour.

C. Persistent infection (PI)

Most PI animals are virus positive and antibody negative, as discussed in section 1.4.5. Consequently, following an initial clinical examination and consideration of herd history, the results of blood tests for BVDV antigen and antibody will confirm PI status. However, final confirmation is obtained by taking two samples three weeks apart (Nettleton and Entrican, 1993). Some PI animals may have low levels of antibody in the ELISA (Horner, 1996). In this report 8 of 41 samples (19.5%) from PI animals that tested positive to the antigen ELISA were also positive to the antibody ELISA.These were submissions to the Central Animal Health Laboratory and were mostly concentrated on a few properties. Without follow up samples from these animals, no conclusions were possible. However, the hypothesis put forward previously (1.5.3) may well apply here if they were confirmed PI cases.

The use of immunohistochemical techniques to detect PI animals is described by Thur *et al.* (1996). They were able to detect BVDV in skin biopsies, and found the test to be a fast and reliable means of identifying PI animals. The results were shown to be accurate when compared with virus isolation or an antigen ELISA (of buffy coat). Results were the same when skin samples were collected from different sites on the animal. The relative ease of this method make it a novel means for the detection of BVDV in carrier

cattle, whether ante- or post-mortem.

D. Mucosal disease

Diagnosis of fatal cases is carried out by virus detection from organs. As death can occur in PI animals due to intercurrent disease, then final proof of mucosal disease requires isolation of the cytopathic biotype from the intestine or other organs (Houe, 1996). As previously described, cytopathic BVDV is almost exclusively isolated from mucosal disease cases (Clarke *et al.*, 1985).

The histopathological diagnosis of BVDV is discussed in Chapters 3 and 4.

The methods for obtaining a herd diagnosis will be covered in the next section under the epidemiology of bovine pestivirus disease.

1.7 EPIDEMIOLOGY

A significant portion of the literature concerning the epidemiology of BVDV comes from the northern hemisphere, where cattle are housed for part or all of the year, and may not be always relavent to temperate climates where stock are permanently at pasture.

1.7.1 Prevalence of infected animals.

BVDV infections appear to be widespread in all cattle-raising countries, although some differences between regions and countries occur (Houe, 1996; Houe, 1999). The prevalence of infection can be expressed in terms of antibody carriers or PI animals. A large number of studies show a somewhat similar prevalence of approximately 0.5% to 2% PI animals and 60% to 85% antibody positive animals (Harkness *et al.*, 1978; Meyling, 1984; Howard *et al.*, 1986; Edwards *et al.*, 1987; Leiss *et al.*, 1987; Hoe and Meyling, 1991; Frey *et al.*, 1996; Braun *et al.*, 1997; Vega *et al.*, 1997).

Four surveys carried out in New Zealand indicate that approximately 60% of all cattle, whether dairy or beef, have neutralising antibodies to BVDV (Golo, 1989; Horner and Orr, 1993; Perez *et al.*, 1994; Perez *et al.*, 1995).

The variation in prevalence in a number of cross-sectional studies has previously been

related to differences in cattle population density, housing systems and management (Houe, 1995). In Canada, the number of cases submitted to the diagnostic laboratories reflects those geographic areas with the highest cattle density (Elves *et al.*, 1996a). If cattle are housed under close confinement, a PI animal can infect more than 90% of other cattle in the herd by the time it has reached 4 months of age (Houe *et al.*, 1993a). The extent of animal trading, the use of common pasturing, and the use of vaccination are management factors that vary considerably among regions, but are difficult to assess due to a small sample size in most prevalence studies (Houe, 1999).

1.7.2 Prevalence of infected herds.

Studies based on the detection of BVDV antibodies, either in individual animals or in bulk milk, indicate that the prevalence of infected herds in most countries is high, in the order of 70% to 100% (Edwards *et al.*, 1987; Houe and Meyling, 1991; Niskanen *et al.*, 1991; Niskanen, 1993; Houe, 1996; Braun *et al.*, 1997; Vega *et al.*, 1997). One notable exception is a report from Finland where only 9 out of 291 dairy herds (3%) had antibodies in bulk tank milk (Niskanen, 1993). See section 1.7.4 for a possible explanation.

Only a few studies have measured the prevalence of herds with PI animals (Houe, 1999). The screening of whole herds in Denmark (19 herds) and USA (20 herds) showed that PI animals were present in 53% and 15% of herds respectively (Houe, 1996). Vaccination had been carried out in 15 of the 20 American herds, but in none of the Danish herds. In a study in Germany, 149 out of 329 herds (45%) were found to contain PI animals (Frey *et al.*, 1996). Following bulk tank milk testing of all dairy herds in Denmark (16,113) it was estimated that 39% of herds had PI animals (Bitsch and Ronsholt, 1995).

From these studies it could be concluded that while most herds (70-100%) have serological evidence of past or present infection, only about half this number of herds (39-53%) are definitely actively infected, as evidenced by the presence of PI animals in their midst. Also, it could be concluded from the screening of the 20 USA herds, that vaccination may be beneficial in reducing the number of PI animals in a herd. The prevalence of BVDV antibody in the 5 unvaccinated American herds reported above was in the expected range (80%), yet the level of PI animals found overall (the 5 unvaccinated plus the 15 vaccinated herds) was much lower (15%) than that recorded in Danish herds (53%) where vaccination is not practised.

1.7.3 Incidence of Infection

At the individual animal level, the annual incidence of risk of infection (or cumulative incidence) has been estimated to be 34% in Denmark (Houe and Meyling, 1991). The incidence of acute infection was calculated from the increase in antibody prevalence from one age group to the next. Then the theoretical risk of foetal infection during the first 3 months of pregnancy was calculated as 3.3%. Some of these foetal infections may have induced abortion or neonatal loss, so this level compared well with the observed prevalence of 2.9% of PI animals among animals younger than one year. Thus, these calculations showed consensus between the established pathogenesis of infection and the epidemiology. Subsequently, cumulative incidence was used in estimating annual losses due to BVDV infections in that country (Houe, 1999). See section 1.7.9 for discussion on economic losses.

At the herd level, a three year study in Denmark revealed new infections in 8 out of 9 herds examined, corresponding to an annual cumulative incidence of 52% (Houe and Palfi, 1993). It was concluded that in areas of high prevalence, where the virus is endemic, the infection status of individual herds can change rapidly (Houe, 1999).

1.7.4 Geographic differences in epidemiology.

In Scandinavian countries the prevalence of infection is greater in areas where cattle density is higher. For example there is higher prevalence in southern Norway and Sweden compared with the northern regions of these countries; and higher prevalence in Denmark compared with Norway and Sweden (Houe, 1996). In addition, herd size increases in the order of Finland, Norway, Sweden and then Denmark, and this is also the order of increasing prevalence. It appears that the rapidity with which new cases develop over a given time period (incidence density) is greater in an area where herds are larger, because once a herd is infected most animals will become infected (Houe, 1996). As described, the vaccination of cattle against BVDV will reduce the prevalence, and Houe (1996) describes the differences between Denmark, where vaccination is not practised, and Michigan, America, where it is widely used. There was a remarkably lower prevalence of infection in Michigan and although vaccination was considered a major factor, as already described, other management factors which reduce infection pressure on pregnant animals were also found to be different between the two countries. For example, the concentration of cattle and cattle herds was lower in Michigan and the

selling of bull calves shortly after birth from Michigan herds reduced the number of PI animals also. It was concluded that if the risk factors could be determined during prevalence surveys in a number of areas, then mathematical modelling could be used to rank their importance (Houe, 1996).

1.7.5 Sources of infection

A. Persistently infected cattle

These are considered the most important source of infection, as the amount of virus excreted is extremely high, and excretion is for a long period of time. Brock *et al.* (1991) showed that serum samples from PI animals diluted by up to 10^6 may still yield virus when examined by virus isolation techniques. In PI animals, virus is continually shed via nasal discharges, saliva, semen, urine, faeces, tears and milk (Coria and McClurkin, 1978). In PI bulls, virus titres up to 10^7 have been found in semen (Paton *et al.*, 1989)

In an experiment where two PI animals were used to transmit infection to susceptible heifers, this was achieved with ease (McGowan *et al.*, 1993). More than half of the seronegative animals (63%) became infected after one day of close contact with a PI cow and her PI calf. This result is consistent with a rate of 60% in 24hrs previously reported by Littlejohns (1985), and demonstrates how readily infection can be transmitted when cattle are yarded with PI herdmates. The rate of spread at pasture may be quite low, in the order of 1% per day (Littlejohns, 1985). This indicates that any yarding of cattle is the time when rapid spread of infection from PI animals occurs.

B. Cattle with acute infection

Following acute infection virus is excreted from day 4 to 10, and sometimes longer (Brownlie *et al.*, 1987). Although the virus is shed in most excretions and secretions, the amount is much lower than from PI animals. Experimental infections in five bulls with acute infection yielded virus titres in semen ranging from 1:5 to 1:75 (Kirkland *et al.* 1991). An example of lower transmission rates is provided by Niskanen *et al.* (1996) where 14 calves were placed in close contact with acutely infected calves for two days and none seroconverrted to BVDV.

C. Other species

BVDV has been isolated from other ruminants, including sheep and goats. Consequently, these are considered potential sources of infection for cattle. BVDV transmission to sheep and back to cattle has been recently demonstrated (Paton *et al.*,

1997). BVDV was transmitted by contact from a PI bullock to pregnant sheep. This resulted in the birth of PI lambs, one of which in turn transmited virus by contact to pregnant cattle. Two of these cows subsequently gave birth to PI calves, from one of which the virus was again transmitted by contact to pregnant sheep, leading to another generation of PI lambs. BVDV has also been isolated from pigs, but their importance as a source of infection to cattle remains unclear (Liess and Moennig, 1990). However, BVDV has been associated with clinical signs of disease in pigs (growth retardation and wasting), which could be a confusing factor in the diagnosis of SFV in countries free from this disease. A survey of the seroprevalence of BVDV in farmed deer in New Zealand found that 9.5% (38 of 400) had antibody (Motha, 2000). In northern Norway, reindeer have been found to have a higher prevalence of BVDV antibody than cattle and sheep, suggesting a reservoir among free-living ruminants (Loken, 1995).

1.7.6 Methods of Transmission

Direct nose-to-nose contact between infected and susceptible animals for one hour is sufficient for infection to occur (Traven *et al.*, 1991). Direct contact with a PI animal is the most efficient mode of transmission of the virus under natural circumstances (Cook *et al.*, 1990; Traven *et al.*,1991; McGowan *et al.*, 1993a; Niskanen *et al.*, 1996). Contact with aborted foetuses is a means of transmission (Littlejohn and Horner, 1990). Venereal transmission may occur, with the PI bull being the greatest risk. Twelve antibody negative heifers all seroconverted after artificial insemination with semen from a PI bull (Meyling and Jensen, 1988). In contrast, when antibody negative heifers were inseminated with semen from an acutely infected bull, only 5% (3 out of 60) seroconverted (Kirkland *et al.*, 1997). At the time of embryo transfer, if the donor cow is a PI, then BVDV is present in high levels in uterine fluids, and will be transmitted with the embryo to the recipient unless proper washing proceedures are performed (Brock *et al.*, 1997). Although faeces containing virus could be a means of transfer, this has not been clearly demonstrated. The time required for inactivation of BVDV in cattle slurry was 3 hours at 35 °C, 3 days at 20 °C, and 3 weeks at 5 °C (Bendixen, 1993). In general, the infectivity of pestiviruses outside the host is of short duration (Hafez and Liess, 1972; Duffel and Harkness, 1985; Liess, 1990). The use of live or contaminated BVDV vaccines has resulted in the transmission of infection (Liess *et al.*, 1984). Airborne transmission is considered possible over several metres (Bitsch and Ronsholt, 1995) but has not been proven and needs further investigation (Houe, 1999). A number

of indirect methods of transmission have been investigated, involving intermediaries that transmit the virus from the infected to the susceptible animal. Iatrogenic spread through the reuse of hypodermic needles, or with nose tongs, has been demonstrated from PI animals, when carried out within 3 minutes (Gunn, 1993). This has also been achieved with the reuse of rectal gloves (Lang-Ree *et al.*, 1994). Blood feeding flies were shown to transmit BVDV from a PI animal to susceptible animals (Tarry *et al.*, 1991).

The practical significance of many of the experimental transmission methods is difficult to evaluate and it appears that the most important means of transmission is direct contact with a PI animal (Houe, 1996; Houe,1999).

The infectious dose of BVDV has been found to vary with the route of administration (Cook *et al.*, 1990). For example, the minimum amount of virus needed to elicit seroconversion in heifers was ten times lower by the intranasal route compared with the conjunctival route.

1.7.7 Transmission patterns

The transmission patterns of BVDV infection between herds and within herds is dependant on a number of factors :-

A. The presence of PI animals

The presence of PI animals constitutes an immense source of infection for susceptible stock. In a dairy herd, more than 90% of the other animals will be infected within 4-6 months if a PI animal is introduced (Houe 1996). Similarly in a study in New Zealand (Golo, 1989) the presence of five PI animals in a herd resulted in all the replacement heifers and 97.1% of the adult milking herd having serum neutralising antibodies to BVDV. The ease with which PI animals transmit infection has been shown experimentally, as previously described. In the field, it has not been established whether the spread of virus is primarily from the PI animal itself, or whether this involves spread from secondary acute infections (Houe, 1999). For transmission of BVDV between herds, the purchase of a PI animal, including a pregnant animal carrying a PI foetus, constitutes the greatest risk (Houe, 1996; Houe 1999). Assuming a prevalence of PI animals of 2%, the calculated risk of introducing infection when 20 animals are purchased is 33% (Houe, 1999). However, it has been shown that infection is

introduced to new herds more frequently than can be explained by the purchase of PI animals alone (Houe and Palfi, 1993). The risk of introducing acutely infected animals was calculated as 8% (based on the assumptions that the annual risk of infection was 30% in a population where 50% of the animals were antibody positive, and that the acutely infected animal was shedding virus for 10 days). In a repeated serological survey of 41 Danish dairy herds, new infections were found to be related to some form of contact with other cattle in most cases (Houe *et al.*, 1997).

The spread of infection within herds in the abscence PI animals has been investigated. In two studies, the virus circulated for 2 to 2.5 years although there were no PI animals present and no direct contact with PI cattle was found (Barber and Nettleton, 1993; Moerman *et al.*, 1993). Where there are no PI animals in a herd the infection can be of short duration (a few weeks) and only include include a small percentage of the herd before transmission ceases (Houe, 1992a). It is possible that the virus could eventually disappear from the population if its potential supply of susceptible hosts is exhausted, as alluded to by Golo (1989). Although there may be variation in the rate at which acute infection spreads in a herd, in most cases a cow in early pregnancy will eventually become infected, a PI calf will be born, followed by rapid secondary transmission to the remaining susceptible animals (Houe, 1999). A common pattern of PI production was seen in 10 out of 22 Danish dairy herds; an initial small group of PI animals, followed by a larger group six months later (Houe, 1992a). Family lines of PI animals are fairly common, and can cause infection to continue for several years (Radostits and Littlejohns, 1988; Houe, 1992a).

B. The management systems

Transmission is promoted by factors such as housing vs grazing, high stocking rate, mating management and manipulations which bring stock into close proximity to one another. It is notable that where animals are housed and pastured in segregated groups, the transmission from PI animals may be prevented until the PI animals come in closer contact with susceptible animals (Houe *et al.*, 1995; Taylor *et al.*, 1997).

C. The strain of the virus

The virulence of the virus may influence the rate and extent of virus transmission. The epidemic of acute disease due to Type 2 BVDV in Ontario in 1993 indicates that highly virulent strains can spread rapidly between herds (Alves *et al.*, 1996a, 1996b).

Similarly, in Britain several cases of acute BVD with severe disease within a few weeks have been reported (David *et al.*, 1994). Within the Type 1 group there is variation in virulence. Those strains that increase susceptibility to respiratory disease may cause coughing among acutely infected animals, with increased dissemination of BVDV (Potgieter, 1997).

The highly virulent Type 2 BVDV strains resulted in outbreaks of an epidemic rather than endemic disease (Houe, 1999). Further epidemiological study of high virulence BVDV strains (both Type 1 and Type 2) is required, as this affects both transmission patterns and financial outcome, as discussed later.

1.7.8 Sequence of clinical manifestations following herd infection.

Houe (1996) reviewed the timing of clinical manifestations following the introduction of BVDV into a herd. Initially, clinical signs of acute BVDV are seen, being immediately followed by repeat breeding for a few months and abortions for several months. PI animals are born 6-9 months after clinical signs of acute BVDV and most calves with congenital defects are born just before the PI animals, and are mostly virus free. Finally, mucosal disease occurs at 6-24 months of age.

Houe notes that it is rare to see all of the clinical manifestations in one herd, and cites the example where many abortions occur in a herd, few or even no PI animals may be born (Houe, 1991). However, one reported case in a New Zealand beef herd showed all of the above manifestations, resulting in a severe loss of production (Frazer, 1997).

1.7.9 Diagnosis of BVDV at the herd level

In herds not using vaccination, the widespread transmission of BVDV from PI animals can be used to indirectly identify herds with such animals, either by serological examination of a few young stock (Houe, 1992b, Houe, 1994) or by determination of antibody levels in bulk milk (Niskanen, 1993; Houe, 1994; Bitsch and Ronsholt, 1995). A small sample of young stock (3 to 10 animals six to eighteen months old) will best predict the presence or absence of PI animals on a farm when either all of the young stock are antibody positive, or almost all are antibody negative (Houe, 1999). Measurement of antibody levels in bulk milk will give an indication of the prevalence of seropositive cows in the herd, and can distinguish totally naive herds from chronically or recently infected herds (Houe, 1999).

1.7.10 Economic losses resulting from BVDV infection

Economic losses arise from a wide range of clinical manifestations, as outlined in Table 1.2, and can be considered at different levels. At the farm level, losses can vary from a few hundred to several thosand dollars (Duffel *et al.*, 1986; Houe *et al.*, 1993b). In the case referred to above (Frazer, 1997) a conservative estimate put the cost of loss of production at NZ $30,000, or $110 per expected calving. Losses involving specific aspects of BVDV infection have been described. For example, reproduction losses have been estimated in New Zealand as NZ $30 to 40 per cow (Woods, 1994) Estimates of economic losses from infection with highly virulent strains have ranged from US $ 40,000 to 100,000 per herd (Carman *et al.* ,1994; Alves *et al.* 1996a, b). An epidemiological study carried out to examine the effect of BVDV infection on the general health of dairy herds showed that the presence of the virus was associated with an increased risk of clinical mastitis, retained placenta, anoestrous and longer calving intervals (Niskanen *et al.*, 1995) making calculation of economic loss a difficult procedure. Calculation of the losses at a national level have been estimated to be in the range of US $10 to 40 million per million calvings (Bennet and Done, 1986; Harkness, 1987; Spedding *et al.*, 1987; Houe *et al.*, 1993a; Houe *et al.*, 1993b; Houe, 1995).

Computerised spread sheet models have been used to calculate losses, and sensitivity analyses showed that the calculations were not significantly affected by variations in many of the parameters used, with the exception of incidence of infection (Houe *et al.*, 1993a, b). Based on an estimated annual incidence of acute infection of 34%, the total annual losses in Denmark in 1992 were calculated to be US $20 million per million calvings. This model was also used to estimate the losses due to the highly virulent strains of BVDV found in Britain and North America (Houe, 1999). At the same incidence used above (34%) the losses due to Type 2 strains were estimated to be US $57 million per million calvings. Low virulence BVDV infections, as seen in Denmark, caused maximum losses at an incidence of 45%, whereas the high virulence BVDV infections caused maximum losses at 65%. It was concluded that the cost-benefit analyses of control programs were highly dependant on the risks of new infections under different circumstances and on the strains of virus involved (Houe, 1999).

Decision tree analyses have also been used to elucidate the economic consequences of different actions in the herd (Houe *et al.*, 1994).

A system of cost benefit analysis of BVDV infection has been outlined by Bennet and Done, 1986, and this remains a sound method of evaluating economic importance today.

1.8 CONTROL

In any cattle population infected with BVDV, only a small percentage of animals (<2%) are persistently infected with the virus. This is reported worldwide, from Scandinavia (Houe,1996) to Australasia (Littlejohns and Horner, 1990). Since the PI animals are the main source of infection both within and between herds, the prevention and control of BVDV infection centres mainly on the elimination of persistently infected cattle (Golo, 1989; Bolin, 1990; Wilks, 1994; Bolin, 1995; Bitsch and Ronsholt, 1995; Cortese *et al.*, 1998).

There are two means of achieving this:-

1. Preventing infection of sero-negative pregnant cattle by ensuring all female stock are seropositive before mating (or insemination) by either vaccination (Bolin, 1996) or controlled natural infection (Horner, 1996).
2. Eliminating infection from the herd by identifying and culling PI animals, and using effective quarantine measures to prevent reinfection of seronegative cattle (Houe, 1996).

1.8.1 Vaccination.

Although BVD viruses differ in biotype and virulence, all are related antigenically and no separate serogroups are defined (Bolin, 1995). Consequently vaccines could be made that would be cross-protective to a wide range of different strains (Bolin and Ridpath, 1990). However, there is mounting evidence that different strains of BVDV may differ in their ability to initiate the various clinical syndromes (Houe 1996), and that some strains give poor protection to the unborn foetus (Cortese *et al.* 1998). Bolin (1996) points out that the emergence of highly pathogenic strains, as well as the finding that the antigenic differences among strains of BVDV was much greater than previously thought, compels a reassessment of the vaccination stratagies required today .

Vaccination has certainly been shown to be protective against clinical disease, such as respiratory disease and acute BVD in calves (Bolin, 1995). However, to break the cycle of ***in-utero*** infection resulting in persistently infected cattle, it is essential that vaccination provides foetal protection, which appears to be much more difficult to achieve. As the mechanism for placental transfer of BVDV is unknown, and only small amounts of virus in the blood stream of the dam appear sufficient to cause development

of immunotolerant calves, protection of the foetus by vaccination is difficult (Cortese *et al.*, 1998). Although commercially available vaccines have been used extensively in some countries, especially the USA, for 30 years, disease associated with BVDV remains widespread (Brownlie *et al.*, 1995). Vaccines licensed in the United States have not been required to provide foetal protection (Campen and Woodard, 1997).

Currently, both modified-live virus (MLV) and inactivated virus is used for vaccine production. Single viral strains are usually used in MLV vaccines, while multiple viral strains are used in inactivated vaccines. Cytopathic strains are used in most MLV vaccines, while several inactivated vaccines are bivalent (contain both CP and NCP strains). Inactivated vaccines incorporate adjuvants to help stimulate the immune response.

A. Inactivated vaccines.

Currently, the most widely used inactivating agent is binary ethyleneimine (Bolin, 1995). The resulting vaccines contain intact killed viruses. Any contaminating adventitious agents are also inactivated by this process. Inactivated virus is unlikely to either induce immunosuppression or alter numbers of circulating lymphocytes within immune cell populations (Atluru *et al.*, 1990; Ellis *et al.*, 1988; Larsson and Fossum, 1992).

Only one inactivated vaccine has been shown to provide adequate foetal protection. This commercial vaccine (called 'Bovidec,'C-Vet) was developed from a Compton prototype, and was shown by Brownlie *et al.* (1995), to confer a high degree of foetal protection. In their first experiment they confirmed the foetopathic nature of the challenge strain of virus (All 9 heifers infected were shown to be viraemic, with one confirmed BVDV abortion, 3 PI calves and 5 calves with active immunity). In their second experiment they vaccinated heifers around the time of mating, and challenged them between 25-80 days of gestation. There was no evidence of a viraemia in the 15 vaccinated heifers after the challenge, nor of any BVDV infection in their calves. All the 6 unvaccinated heifers showed evidence of infection (three heifers became viraemic, another aborted, with BVDV being recovered from foetal tissues, and four calves were born as PI). Of the 15 vaccinates, nine were vaccinated three times to achieve these results, and it could well be inferred from the antibody response to vaccination that

three doses may be needed for this vaccine to provide protection to the foetus. It was further implied that the amnestic antibody responses seen after the second and third vaccinations suggested that the Bovidec vaccine can stimulate cell-mediated immune mechanisms in addition to humoral antibody. When the two parts of this experiment are considered from the viewpoint of foetal infection, there was 100% protection to 15 foetuses out of 15 vaccinated animals, whereas there was evidence of *in-utero* infection in 14 out of 15 foetuses in the unvaccinated control animals. Brownlie *et al.* (1995) claimed that this describes a good model for the validation of vaccines designed to protect the bovine foetus from BVDV.

The main disadvantage associated with inactivated vaccines is the requirement for two doses to achieve primary immunisation, and the need for annual boosters. One study indicated that neutralising antibody persisted for less than one year (Bolin *et al.*, 1991a). The greater cost of production (compared to MLV vaccines), along with the extra handling required, is another disadvantage. Maternal antibody may interfere with inactivated vaccines in calves up to 8 months of age (Horner, 1996).

B. Live Vaccines

The MLV vaccines used in North America usually contain a single strain of cytopathic BVDV (eg NADL, Singer or C24V) attenuated through repeated passage in bovine or porcine cells (Coggins *et al.*, 1961; Phillips *et al.*, 1975). A vaccine used in Europe contains a chemically modified virus. This mutant is temperature sensitive, replicates poorly in cows and is safe for use in pregnant cows (Lobmann *et al.*, 1984; Lobmann *et al.*, 1986).

Advantages of MLV Vaccines

The live virus replicates in the vaccinate, so only small numbers of infectious viral particles are required for immunisation (Kahrs, 1966). This makes MLV vaccines inexpensive. A single dose of vaccine is sufficient for immunization, and the duration of antibody is probably similar to that elicited by field virus during a natural infection (Bolin, 1995). Immunization of calves with MLV vaccine is not inhibited by colostral antibody at viral neutralising titres up to 1:32 (Menanteau-Horta *et al.*, 1985). Based on a half-life of 21 days for colostral antibody and an initial viral neutralizing antibody titre of 1:2000 after ingestion of colostrum, it has been estimated that immunization with live vaccine should be successful in most calves that are 4 to 6 months of age (Schultz,

1993).

Disadvantages of MLV Vaccines

As with any live vaccine, if it is stored or handled incorrectly, failure of immunisation can occur. The risk of vaccine contamination with an agent that is capable of causing disease is a further disadvantage. For example, foetal bovine serum used in cell culture systems for vaccine production are frequently contaminated with field strains of BVDV (Kniazeff *et al.*, 1975; Nuttal *et al.*, 1977; Rossi *et al.*, 1980; Potts *et al.*, 1989; Bolin *et al.* 1991b; Vilcek *et al.*, 1998). In New Zealand, a Type 2 BVDV was found as a contaminant in foetal bovine serum of American origin (Vilcek *et al.*, 1998) indicating a high risk for the spread of severe forms of BVDV infection through live vaccines. There are reports of outbreaks of disease that followed the use of vaccines contaminated with noncytopathic BVDV. For example, BVDV infections have been detected in piglets born to sows vaccinated against swine fever with contaminated vaccines (Wensvoort and Terpstra, 1988). Pestivirus has been reported in human viral vaccines (Harasawa and Tomiyama, 1994). Low levels of neutralising antibodies to BVDV were found in 16% of people in a survey to investigate the possibility of BVDV infection in occupational groups at risk (Wilks *et al.*, 1989). There was no clear association with occupational contact with cattle, indicating that exposure to BVDV by contact with infected animals was probably not the cause of the neutralising activity detected. The possibility of BVDV infection through contaminated human viral vaccines needs to be further investigated.

Other disadvantages associated with MLV vaccines include adverse reactions to vaccination. Firstly, foetal infection was shown to occur after the vaccination of pregnant animals (Liess *et al.*, 1984). This can result in reproductive failure, foetal death and congenital defects, so MLV vaccines are not recommended for use in pregnant cattle. Secondly, the induction of mucosal disease when cytopathic strains were used in the vaccine was first described by Peter *et al.* in 1967. Postvaccinal MD was reported at that time as an infrequent, highly fatal condition occurring 1-4 weeks after vaccination (Bittle, 1968; Clark, 1968; McKercher *et al.*, 1968, Rosner, 1968). However, it was not understood that this was due to superinfection of existing PI animals with the CP strain until Brownlie's work was carried out in 1984. A subsequent study showed that some PI animals can survive vaccination without adverse effects (Bolin *et al.*, 1985b). However these cattle are not protected from a subsequent infection by a cytopathic BVDV that is antigenically different from the vaccine virus, but similar to the infecting NCP virus,

with fatal consequences. Thirdly, an increased mortality rate in beef calves, thought to be related to vaccine virus-induced immunosuppression has been reported (Roth and Kaeberle, 1983). This immunosuppression probably enhances the pathogenicity of other infectious agents, resulting in postvaccinal disease. Finally, there is a potential for MLV vaccines to undergo genetic recombination with the nucleic acid of other viruses or of the vaccinate. The RNA of BVDV has been shown to recombine with RNA of both cellular and viral origin (Meyers *et al.*, 1991; Meyers *et al.*, 1992; Qi *et al.*, 1992). This recombination can create a virus capable of causing disease (Bolin, 1995).

Despite these problems, it is widely recognised that a modified live BVDV vaccine may be the best means of immunising female cattle before breeding, and so prevent infection of the foetus in early gestation (Bolin, 1996). Ideally, the PI animals would be first identified and removed before vaccination took place to avoid postvaccinal MD. As with inactivated vaccines, MLV vaccines have been expected to protect against clinical disease without knowledge of their ability to protect against foetal infection (Bolin, 1995).

Following the model described by Brownlie *et al.* (1995), a MLV vaccine has now been shown to confer adequate protection to pregnant heifers and their foetuses ('Resvac 4', Pfizer) following a single dose administered intramuscularly (Cortese *et al.*, 1998). This was a combination vaccine containing a modified-live, cytopathic, type 1 BVDV (NADL strain). They first checked the effectiveness of their challenge strain (NCP, type1, BVDV), and were able to achieve 100% infection, with all heifers (6) producing PI calves. The vaccinated animals (12) were then challenged. All 12 heifers remained negative for virus isolation. Ten of these vaccinated heifers developed high levels of antibody 30 days after exposure (titres > 640) and their calves did not become PI. Another two heifers showed a lower immune response at this time (titres of 160) and produced calves that were considered persistently infected. This gives a success rate for vaccination of 83.3%. The challenge dose did not seem excessively high ($5x10^5$ $TCID_{50}$), so perhaps a booster dose of this live vaccine would have ensured complete protection, as alluded to by Bolin (1996).

C. Autogenous vaccines

Another means of providing protection is the use of autogenous vaccines (Horner, 1996). Once a PI animal is identified on a property, serum from this animal is used to

innoculate other cattle on the farm. This was used over a number of years in heifers in a government-owned herd in New South Wales, Australia, where neat serum from a PI herdmate was inoculated intranasally (Littlejohns and Horner, 1990). They also report unpublished data where serum diluted up to 1/1000 and injected subcutaneously was successful in inducing seroconversion and interrupting the sequence of PI births and disease in the herd (Cook LG, Jessep TM and Littlejohns IR, unpublished data) The disadvantages of this crude vaccination method include the inadvertent transmission of other blood-borne diseases such as bovine leukaemia and bovine immunodeficiency virus. In addition, there is the possibility that overt disease could occur, especially if susceptible pregnant cattle are inadvertently infected.

D. Future vaccines

Since it has been established that two genotypes of BVDV exist, that have distinct antigenic characteristics (Ridpath *et al.*, 1994), new vaccines will likely contain viruses from each genotype.

In the future, the proof of vaccine efficacy must shift from the prevention of acute disease to the prevention of foetal infection (Dubovi, 1992; Bolin, 1995) An example of the problems that exist here is given by the Wyoming State Veterinary Laboratory, USA. They deal with evidence of foetal disease attributable to BVDV in well-vaccinated beef herds, and point out that not one of the BVDV vaccines on the market is labelled for foetal protection (Campen and Woodard, 1997). They indicate that lawsuits unfairly directed against practitioners for "vaccine failures" have occurred after proper administration, and express concern that foetal infection may not be preventable with the current BVDV vaccines.

New biotechnology may lead to the production of a new generation of vaccines. For example, subunits of BVDV (partially purified proteins) have been used to make immune-stimulating complexes (ISCOMs) which stimulate high titres of viral neutralising antibody (Kamstrup *et al.*, 1992). Another example is the use of genetic engineering techniques to produce new vaccines. Recombinant DNA technology has been used to incorporate nucleic acid sequences from swine fever virus into pseudorabies virus (Van Zijl *et al.*, 1991). Pigs immunised with this recombinant virus were protected against both pseudorabies and swine fever. A group working at the Animal Research Unit, Moorooka, Queensland is producing a recombinant vaccine for use in their beef industry (personal communication, Peter Kirkland). They are using a

herpes virus as a vector, into which they are inserting a highly antigenic part of the BVDV genome. With the new knowledge of the antigenic diversity of BVDV, as well as the understanding that certain strains are associated with different aspects of the disease, vaccines containing a wide variety of strains may be used in future.

Finally, improved delivery technologies such as slow or pulse release could enhance the efficacy of vaccines (Wakelin, 1993).

1.8.2 Controlled exposure to PI animals.

It is possible to use known PI animals to infect susceptible cattle (Horner, 1996). Success depends on the quality of contact between the PI animals and the cattle to be infected. Close contact means yarding rather than a grazing situation. In one study 62% of susceptible cattle were infected overnight by yarding with a PI animal, whereas transmission under grazing conditions was about 1% per day (Littlejohns, 1985). Great care is required to ensure that pregnant animals are not exposed. However, considering the number of reports of inadvertant infection in trials carried out in presumed isolation, the use of controlled exposure at the farm level probably poses far too great a risk to other stock to make this a feasible method of protection.

1.8.3 Elimination of infection.

A. Artificial Insemination (AI) Centres.

As BVDV is excreted in semen in both acutely infected and PI bulls, the avoidance of entry of infected bulls to the AI centre is imperative, as well as the detection and elimination of any bulls found to be shedding BVDV in their semen. Detection of infected bulls can be achieved by testing twice for virus in the blood before final entry to the AI centre (Houe, 1999). There is always the risk that an early acute infection may escape both virus and antibody detection tests, so a period of isolation at the AI centre is required. The initial test for virus can take place on the farm, and the second test during this period of isolation. In addition, virus may continue to be shed in semen after the end of a viraemia (Kirkland *et al.*, 1991) and persistent shedding of BVDV in semen has been observed in one bull in the absence of viraemia (Voges, 1997). This makes it imperative that regular testing of bulls for virus in their semen is conducted at the AI centre, along with monitoring for seroconversion (Houe, 1999).

B. Eradication of BVDV in Scandinavia and Finland.

In 1993, national control initiatives for the eradication of BVDV started in Sweden and Norway, and in 1994, in Finland and Denmark (Alenius *et al.*, 1997). These counties had not used any vaccine to control BVD. The control of BVDV without the use of vaccines is based on three approaches: (1) the identification of herds with active infection; (2) the clearance of virus shedding [PI] animals from the herds; and (3) the implementation of control measures that will prevent both infection of herds already free of the disease and also reinfection of herds cleared of the disease (Bitsch and Ronsholt, 1995). Firstly, the development of an indirect ELISA test ("Svanovir") that could accurately determine the levels of antibodies to BVDV in bulk milk samples enabled infected and uninfected herds to be discriminated (Niskanen, 1991). A positive relationship was found between the prevalence of antibody in cows and the absorbance values for bulk-tank milk samples (Niskanen, 1993). Secondly, it was shown that it was possible to determine if a herd contained PI animals by blood testing 5 animals aged 6-18 months. If two or more were positive for antibody, then there was a greater than 98% probability that the herd had PI animals (Houe, 1991). This is based on the principle that PI animals will spread infection to almost all the animals in the herd. Individual testing of animals in these suspected PI herds allowed identification of the PI cattle and their removal from the herd, the basis for the eradication procedure. Each country developed a number of milk and blood testing systems to suit their eradication programs.

Progress in Sweden:-

The eradication program was initiated at the request of the farmers in 1993 and was voluntary, with most of the costs being carried by the farmer (Hult, 1997). Twenty three percent of 18,202 herds were negative at that time (Forshell *et al.*,1994; Niskanen, 1993) and by 1997, 55% of dairy herds had been declared free (Hult, 1997). The biggest difficulty was in the organising of BVDV testing of sale animals to avoid the introduction of PI animals into seronegative herds (Hult, 1997). In addition, special auctions were arranged for sale of animals from herds declared free from BVD. An interesting finding was that calves sold free from BVDV were found to have a higher growth rate and a reduced need for antibiotic therapy.

Progress in Norway:-

In the first screening of all 26,430 dairy herds 63% were antibody negative and 9% had PI animals (Waage *et al.*, 1994), while by 1997, 74.3% were antibody negative (Nyberg and Waage, 1997). BVD is a notifiable disease in Norway, with restrictions being

placed on infected herds to prevent further spread (Bitsch and Ronsholt, 1995).

Progress in Finland:-

Only 9 of 291 dairy herds (3%) were found to contain antibodies in bulk tank milk (Niskanen, 1993). Subsequent testing of 34,115 herds showed that only 1% had BVDV antibodies (Kulkas, 1997). This remarkably low figure is difficult to explain, but may relate to the isolation of Finland from the other Baltic countries. The program introduced was a voluntary one. Herds are blood tested in connection with a bovine leucosis program, in which beef cows are included. The results from Finnish dairy herds indicate that they will be free from BVDV in the not too distant future. (Kulkas, 1997).

Progress in Denmark:-

The situation in Denmark was the direct opposite to Finland, as very few dairy herds were initially seronegative to BVDV (Bitsch and Ronsholt, 1995). A survey based on a serological screening test of 10 young stock per herd revealed that almost all the herds had antibody positive animals in the sampled group, and 43 % probably contained PI animals (Houe, 1994). Because of this very high prevalence, it was first decided to examine whether eradication was possible with the tests available. A control and eradication project on the island of Samsoe, involving 36 dairy herds and 75 beef herds started in early 1992 (Bitsch, 1997). Initially, all animals > 3months of age were blood tested for virus and antibody, and all PI animals were removed. Farmers were taught general infection prophylaxis and control measures to prevent the introduction of PI animals. Follow up blood tests in 1993 showed that spread of infection had ceased and that the new ELISAs for antigen and antibody were reliable (Bitsch, 1997). A second study was then implemented on Samsoe island to evaluate the test systems needed to monitor the infection status of herds. It was found that the antibody ELISA used on bulk tank milk gave excellent clarification of the infection status in a part of the dairy herds, while a blood test of just three young animals over 8 months of age using the two ELISAs could be used for the same purpose in all other herds with acceptable reliability. A voluntary national control and eradication scheme was initiated in 1994, and a number of testings arranged. In 1994, an initial bulk tank milk test of all 16,113 dairy herds in Denmark (average of 42 cows per herd) showed that approximately 39% had PI animals (Bitsch and Ronsholt, 1995). By 1997, of 12,311 dairy herds, those with PI status had reduced to 24%, while the herds free of the disease had risen to 68%, and 8% remained unclarified (Bitsch, 1997). At the same time, of 22,879 beef herds, 57% had a free status, 10% a PI status, while 33% remain unclarified.

The primary aim of these programs was to firstly identify and protect the herds free from infection, and secondly to gradually reduce the number of infected herds. One measure of progress is the number of herds seronegative to BVDV, and this has been tabulated below from the data described. (Table 1.3)

Country	Seronegative Status		Total number of Herds	Ave. no. of Cows per Herd
	1993/1994	1997		
Norway	63%	74.3%	26,430	14
Sweden	23%	55%	18,202	26
Finland	97%	>99%	34,115	12
Denmark	<5%	68%	16,113	42

Table 1.3 Progress of Eradication Programs in Dairy Herds in Scandinavia and Finland.

The progress to date is quite reasonable, but as the program continues some problems could arise. All programs are based on the premise that detection and culling of PI animals will achieve control of the disease. However, the BVD virus can continue to circulate in the absence of PI animals; up to two years in one study (Houe, 1996). In another study, the number of new introductions into herds was greater than could be explained by purchase of PI animals, so other unidentified means of introduction can be involved (Houe and Palfi, 1993). Other ruminant species have been shown to transmit the disease (Paton *et al.*, 1997) and this could interfere with the eradication proceedure. Denmark take this into account in their infection prophylaxis proceedures. They recommend that on infected farms small ruminants should be included in examination proceedures. This does not cover the wild ruminants such as deer, however.

The small herd size in Scandinavia (Norway, Sweden and Denmark) and Finland (mean of 24 animals) makes the implementation of an eradication program more economic. The cost of the control and eradication program in Denmark over the first 3 years has been in the order of 40 million NZ dollars, while the losses due to BVDV infection over that period have been estimated to be 75 million NZ dollars (Bitsch and Ronsholt, 1995).

1.8.4 Control of BVDV in N.Z.

On overview of the disease in New Zealand will be given before the control measures are described.

History

The first severe MD-like syndrome seen in N.Z. was reported in 1961, and although cytopathogenic agents were isolated, these were not confirmed as pestiviruses, and prominent kidney lesions suggested a different diagnosis (Salisbury *et al.*, 1961). The first presumptive evidence for the presence of BVDV came from a serological survey, where neutralising antibodies to BVDV were reported in cattle (Fastier and Hansen, 1966). The following year, virus isolation from a clinical case of acute MD confirmed the presence of the disease in N.Z. (Jolly *et al.*, 1967). The reported clinical signs, necropsy findings and histopathology were those typical of MD today, and a cytopathic strain of BVDV was isolated. Acute mucosal disease was then further described (Horner and Buddle, 1970). Serological evidence of BVDV was found in cattle and sheep in the South Island (Robinson, 1971). Further studies of antibody levels in cattle in the 1970's established that serological evidence for BVDV was in the order of 30-35% (Durham and Forbes-Faulkner, 1975; Durham and Burgess, 1977). More recent investigations into the prevalence of neutralising antibodies to BVDV indicate that this is about 60% (Perez *et al.*, 1994; Perez *et al.* 1995). The extent of herd infection is thought to be about 85% (Horner, 1996). MD was notifiable in the 1960's due to its resemblance to serious exotic diseases such as Foot and Mouth Disease. However once its aetiology was determined and it was established that the virus was widespread, it was removed from the list (Littlejohn and Horner, 1990).

Disease syndromes

A number of case reports and laboratory investigations have associated BVDV infection with a wide range of clinical entities encountered in New Zealand. Early reports associated the disease with foot lesions (Mossman and Hanley, 1976) and polyarthritis (Hanley and Mossman, 1977), enteritis in autumn and winter, pnuemonia, concurrent parasitism and bacterial infections such as *Dermatophilus* and *Salmonella* (as reviewed by Littlejonn and Horner, 1990). A high incidence of congenital defects and stillborn calves over four years in a large beef herd was attributed to the BVDV (Gill, 1989). Losses due to MD were more common in the chronic form (Littlejohns and Horner, 1990). Most of the possible clinical manifestations of the disease were reported when

two beef properties suffered infection (McNeil and Van der Oord, 1993; Frazer, 1997). A case of illthrift was documented by Vermunt and Bruce (1993). Pestivirus infection is now one of the main reported causes of illthrift and diarrhoea in cattle in N.Z.; one quarterly surveillance report indicated that of 113 confirmed cases of "illthrift/diarrhoea", 36 were due to pestivirus infection, 35 due to *Mycobacterium paratuberculosis*, 25 to *Yersinia* species, 15 to trace element deficiency and 2 to gastrointestinal parasitism (Anonymous, 1997). Serological evidence indicates that BVDV is one of the viruses responsible for bovine respiratory disease (Motha *et al.*, 1997). The bovine pestivirus has recently been described as an important cause of reproductive failure in heifers (Pickering, 1999).

BVDV in other species

Serological evidence of BVDV infection in deer was reported in 1981 (Anon., 1981), while more recently, examination of intestinal sections of a red deer with weight loss revealed lesions highly suggestive of pestivirus enteritis (Hutton *et al.*, 1999). An outbreak of disease in farmed fallow deer in Sweden was reported which may have involved BVDV infection, possibly in conjunction with another virus (Diaz *et al.*, 1988).

Molecular characterisation of ovine pestiviruses from Britain, Sweden and N.Z. indicate that sheep can be naturally infected not only with border disease (BDV) but also with BVDV, both types one and two (Vilcek *et al.*, 1997). This is of significance with respect to disease control, as it appears that cross-infection between sheep and cattle can occur.

Nucleotide sequencing has been conducted on BVDV strains isolated in Europe, North America and New Zealand. The results indicate a genetic heterogeneity within certain coding regions, whereas the derived amino acid sequence of all BVDV strains was identical and yet differed from that of SF viruses (Hertig *et al.*, 1995; Stadler *et al.*, 1996). This means that PCR technology now allows differention between bovine and porcine pestiviruses. This is important because the early signs of SF, a disease exotic to N.Z., can resemble BVDV infections in pigs.

It was also suggested that genetic heterogeneity in the BVDV group may result from passage in transiently infected animals (Hertig *et al.*, 1995).

Control

A live attenuated vaccine ("Bovax", TVL) was made from the original CP virus isolated in New Zealand by Jolly *et al.* (1967) and was sold throughout the 1970's before being withdrawn due to low sale volumes (Wakelin,1993). Imported inactivated

vaccines became available in N.Z., but these were only provisionally licensed, as no data was available on the efficacy of these vaccines here (Wakelin, 1993). The situation remains the same today; the issue of antigenic variation poses a question about their efficacy, their duration is unknown, and their ability to protect the foetus has not been tested. This makes it difficult for the practitioner to promote vaccination as a viable control measure.

In AI centres in N.Z., all bulls are screened for BVDV before entry, are quarantined after entry, and semen tested prior to use for artificial breeding (Voges *et al.*, 1998). These measures should be successful in preventing transmission of BVDV at the time of AI.

BVD associated infertility problems were avoided by using only BVD antigen negative and vaccinated bulls for natural mating of heifers (Pickering, 1999).

Following his study, Golo (1989) concluded that no specific, reliable, or practical control measures could be recommended in N.Z., but that losses could be minimised by ensuring replacement heifers had high titres of antibody before the breeding season. He suggested exposure to PI animals under natural conditions or vaccination with inactivated vaccines at 12 months of age.

Options for control were further described by Wilks (1994). He concluded that even though there was a very high prevalence of natural infection, there still existed herds that were naive with respect to BVDV infection. Since these naive herds suffer the most severe manifestations of disease following introduction of BVDV, the first task may be the provision of testing to determine herd status. This would form the basis for subsequent advice on selection and screening of replacement animals, and the value of prophylactic vaccination for the herd.

The exact extent of losses due to BVDV infection in New Zealand is not known, but nationally this disease is probably a significant cost to the cattle industry, both dairy and beef.

HISTORY AND OBJECTIVES OF THIS THESIS.

A pestivirus disease outbreak in calves in the province of Taranaki in New Zealand was brought to our attention by Dr G. W. Horner, Chief Veterinary Virologist for the Ministry of Agriculture and Fisheries (Central Animal Health Laboratory, Wallaceville Research Centre, Upper Hutt). This outbreak occurred during the 96/97 dairy season, and, at the time of notification, 38 calves had died out of a mob of 102. Clinical assessment by local veterinarians followed by laboratory testing resulted in a diagnosis of MD. This outbreak had, however, some unusual features. The herd mortality of 37% was higher than than expected for MD. Deaths were occurring in cattle less than six months old. All clinical cases progressed to a fatal outcome, and the outbreak was still continuing.

Blood testing had been carried out at the farm during January, February and March 1997. The BVD antigen ELISA had been conducted on blood samples from 92 calves. Twenty six calves had tested positive for antigen, and seventeen of these had since died. Of the nine remaining antigen positive calves, five were also positive for BVD antibody, which raised the possibility that there may have been deaths associated with acute infection as well as MD, possibly even Type 2 BVDV infection, which had hitherto not been diagnosed in New Zealand.

A visit was made to the farm on the 24th April, 1997, where it was arranged for the nine remaining BVD antigen positive calves to be donated to Massey University for further investigation. The blood tests were repeated on these calves, they were euthanased and necropsied and virus isolation in tissue culture attempted.

If virus was isolated, then its virulence would be tested by inoculation into naive calves which would be subjected to the above examinations.

The main objective was to determine whether this was a new strain of pestivirus capable of causing fatal disease in its own right, as occurs with Type 2 strains. Another important objective was to determine whether a locally available vaccine ("Mucobovin", Merial) would provide protection against this strain.

CHAPTER TWO

GENERAL METHODS AND MATERIALS.

2.1 COLLECTION AND PROCESSING OF BLOOD SAMPLES.

Blood samples were collected from the jugular vein with the calf restrained in a head bale. Blood was collected into 10 ml evacuated glass tubes (Vacutainer, Becton Dickinson, Franklin Lakes). The type of tube chosen was dependent on the use :-

For virus isolation from buffy coat - *Heparin* (green code).

Heparinised bloods were processed on the day of collection. After centrifugation at 600 g for ten minutes, the buffy coat (approximately 1ml) was removed and stored at -70 ^{0}C until tested.

For haematology & antigen ELISA - *EDTA* (purple code).

EDTA blood samples were delivered to the haematology laboratory within 2 hours after collection, where smears were prepared, and tests were carried out on the same day. Whole blood samples for antigen ELISA testing were sent to the CAHL in Wallaceville, Upper Hutt, that same day.

For antibody (ELISA or SNT) - *Plain* (red code).

Blood samples were first allowed to clot at ambient temperature for 4 hours, and then placed at 4 ^{0}C for 20 hours to allow expression of serum. Following centrifugation at 600 g for 10 minutes, the serum was removed and 2 ml aliquots dispensed and stored at -20 ^{0}C until tested.

2.2 TRANSPORT, HOUSING AND FEEDING OF CALVES.

The calves from the outbreak and for the transmission experiment were transported from Opunake and Riverside Farm, respectively, to Massey University and placed directly into the large animal isolation facility. Straw was used as bedding in the two pens, and feed was ad lib meadow hay and calf pellets fed twice daily, along with fresh water. A 'deep litter' system was used for the calves from the outbreak, whilst special yards adjacent to the isolation ward were used to allow 'mucking out' of pens every three days during the transmission experiment. These yards were cleaned and

disinfected with a phenolic preparation (Phensol, Tennaco Organics, Bristol) after each use.

2.3 EXAMINATION OF CALVES.

A complete clinical examination took place in a crush with a head bale, allowing a thorough check of all body systems. Installation of a portable crush within the isolation ward facilitated handling of the calves throughout the transmission experiment.

2.4 EUTHANASIA OF CALVES.

The nine calves from the outbreak were euthanased with 20-30 mls of sodium pentobarbitone (500 mg/ml - Pentobarb 500, Chemstock Animal Health Ltd, Christchurch, N.Z.) administered as a single bolus intravenously. With the eleven calves in the transmission experiment, half this rate was used, to first achieve anaesthetic relaxation, unconciousness and analgesia, which was followed by exsanguination (by severing the jugular veins and carotid arteries). This was carried out in order to avoid the congestion of organs which is a common artefact when pentobarbitone alone is used.

2.5 NECROPSY.

Post-mortem examination was carried out using the standard technique taught at Massey University (Refer printed notes for course no. 116.406). Gross lesions were described, and samples collected for histology and virology. The range of samples collected is described in Appendices 3.1 and 4.1. In addition, some selected samples were collected for bacteriology and parasitology. Tissues for virology were collected first where possible. Tissues for histopathlogy were fixed in 10% formol saline, processed into paraffin, sections cut at 4 μm and stained with haematoxylin and eosin (H&E). Sections were scanned under low power (4 times objective) and any lesions found were further examined under higher power (10 & 40 times objective) and the results recorded, along with the morphological and final diagnoses. The tissues examined were scored 0 to 4 to indicate the presence of a lesion and the degree of severity [0 = no lesion; 1 = mild lesion; 2 = moderate lesion; 3 = severe lesion; 4 = severe and widespread lesion].

2.6 VIROLOGY.

2.6.1 Cell culture techniques.

Primary bovine embryonic lung (Belu) and Madin Darby Bovine Kidney (MDBK) cells were used. Initially, the Belu cells were used for virus isolation and propagation, while the MDBK cells were used mainly for SN testing. The cells were obtained from a bank held at this laboratory and stored under liquid nitrogen at -196 ^{0}C. Later in the research project, a batch of known BVDV-free MDBK cells were imported from the Veterinary Institute of Agricultural Science (VIAS), Melbourne, Australia because of their ease of growth in microtitre plate systems in comparison to Belu cells. This facilitated the immunoperoxidase staining of the NCP strain used in the transmission experiment.

Preparation of cell culture media.

Growth Medium (GM) was Eagles Minimum Essential Medium (E-MEM, Sigma, U.S.A.) with equine serum (ES) added at 15% for Belu cells and 10% for the MDBK cells. For Maintenance Medium (MM) the ES concentration was reduced to 5% for both cell lines. In addition, an antibiotic solution (PSK, Gibco, Auckland, N.Z.) was added at 1%.

Further requirements for the Belu cell cultures were an amino acid solution (BRL, Gibco, Auckland, N.Z.) added at 1%, and Tryptose Phosphate Broth (TPB, Diffco, Detroit, Michigan, USA) which was added at 10%.

See Appendix 2.1 for a detailed description of the reagents used in cell culture.

Initiation, maintenance and passage of cell cultures.

All work with cell cultures was carried out in a laminar flow cabinet ("Nu Aire", Model No. Nu-201-430, Manufactured 1991, Plymouth, U.S.A.).

After removal from liquid nitrogen, the cells were thawed rapidly, resuspended in E-MEM, centrifuged, and the supernatant discarded, to remove the dimethyl sulphoxide (DMSO) used in the freezing down process. The pellet was then resuspended in GM, the cells counted and seeded at a concentration of 2×10^5 cells/ml into 80 cm^2 plastic tissue culture flasks (Nunclon, Denmark) and incubated at 37 ^{0}C in a 5% CO_2 atmosphere.

The method used for counting cells involved initial gentle shaking of the cell suspension for 2-3 seconds, removing a 20 ul aliquot and diluting this 1:5 in Trypan blue solution.

Viable cells were counted in a Neubauer haemocytometer. The number of cells in the original suspension was then calculated, and by diluting with GM, the desired concentration of cells achieved.

Once confluent monolayers were attained (within 24-48 hours) the GM was replaced with MM.

Cells were passaged every 4-5 days. The media was discarded, and monolayers washed gently, twice, with 3-5mls of sterile phosphate buffered saline (PBS), pH 7.2. Two to three mls of Antibiotic-Trypsin-Versene (ATV) solution, containing 0.05% trypsin, was placed on the monolayer and incubated for 2-12 minutes at 37 ^{0}C depending on time to visible disaggregation. The flask was then vigorously tapped to separate aggregates of cells before the addition of 7-8 mls of GM. This 10 ml suspension of cells was then pipetted three times to further separate the cells before counting, as described above.

2.6.2 Virus isolation

Buffy coat samples.

Belu cells at passage level 5 to 10 were used to isolate virus from buffy coat samples from the calves from the clinical outbreak. Cell monolayers were established in 25 cm^2 tissue culture flasks. Once monolayers were about 90% confluent, the GM was discarded and 800 µl of each buffy coat sample was added to each flask and incubated at 37 ^{0}C for one hour. Then 6 ml of MM was added and the flasks incubated at 37 ^{0}C in 5% CO_2. Two control samples were treated in the same manner - the negative control had no sample added, while a known cytopathic strain (NADL) was used as the positive control. After four days the plates were examined under a phase contrast microscope for possible CPE. The flasks were then frozen and thawed and the supernatant centrifuged (600 g for 10 mins). Then 500 µl amounts were transferred to fresh monolayers in 25 cm^2 flasks. One further passage was performed. Cell cultures which failed to show CPE after three passes were considered negative for cytopathic BVDV. Samples of supernatant from the second passage were sent to the CAHL to test for BVDV antigen using the ELISA test.

Tissue samples.

MDBK cells (from VIAS) were used to isolate virus from tissue samples collected from calves in the transmission experiment. The samples tested were spleen and lymph node. The MDBK cells were grown in 24-well tissue culture plates (Falcon, Becton Dickinson, New Jersey, USA). With each sample, the tissues were minced, ground,

suspended in E-MEM, lightly centrifuged, frozen to -70 ^{0}C, thawed , resuspended and recentrifuged. The suspension was filtered using 0.2 μm filters (Micro Filtration Systems, California, USA). A 250 μl aliquot of supernatant from each sample was placed in each well of the tissue culture plate and adsorbed for 30 minutes. GM was added to each well to make a total of 2 mls before incubation. Two further passages were carried out at 4-day intervals. For the third passage, a 96-well plate was used and six duplicates of each sample was made. To each well was added 100 μl MEM, 20 μl of innoculum from the second passage and 100 μl of MDBK cells. Finally, the isolation plate was fixed (after 4 days) using a 20% acetone solution (see Appendix 2.1). This involved washing each well with 100 μl 0.5 M saline, before adding 200 μl acetone fixative for 10 minutes, draining and placing in a 37 ^{0}C drying room overnight. Plates were sent to the CAHL for IP staining.

Serum samples.

Virus isolation from serum samples was carried out with calves from the transmission experiment. Samples were sent to the CAHL for a two-pass test and IP staining.

2.6.3 Virus Neutralisation test

Materials

MDBK cell cultures were initiated and maintained as already described in 2.6.1.

Growth Media was prepared as described in 2.6.1 and stored at 4 ^{0}C, but it was warmed to 35 ^{0}C before use.

The NADL cytopathic strain was obtained from our bank at Massey University. Working stock was prepared by passage of this virus in MDBK cell culture, the titre determined, and aliquots stored at - 70 ^{0}C.

All serum samples were stored at -20 ^{0}C, and before testing were heat inactivated by incubating at 56 ^{0}C for 30 minutes.

Methods

The titre of virus in cell culture was determined by making serial tenfold dilutions of the stock virus suspension in GM and adding an equal volume of cell suspension. This was performed in 96-well tissue culture micro-titre plates (Nunclon, Denmark) using 100 μl volumes per well of each dilution (from undiluted to 10^{-1} through to 10^{-9}). Six replicates were made of each dilution. An equal volume of cell suspension (100 μl) was then

added to each well. A row of control wells was included, where only cells were added (ie 100 µl of cell suspension and 100 µl of media).

The plate was incubated in a 5% CO_2 atmosphere at 37^0 C for 5 days.

The wells were scored for the presence of CPE, and the titre calculated by the Karber-Spearman method (Lennette and Schmidt, 1979).

The procedure used for the SNT was as follows :-

Using 96-well tissue culture micro-titre plates, GM was first added to wells to allow serial dilutions of test sera, preparation of cell and serum controls, as well as dilution of stock virus in order to carry out a back titration to confirm its titre.

Twofold serial dilutions of each serum sample were made in duplicate, from 1:2 up to 1:4096. This was started by first adding 50 µl of each test serum to 50 µl of GM. Cell culture microtitre tips were changed at each dilution. Specific volumetric instruments (Pipetman, Gilson Medical Electronics, France) were used according to the manufacturers recommendations to dispense the required amounts.

The titred stock virus was diluted in media to achieve a rate of 100 $TCID_{50}$ per 50 µl. An equal volume of virus suspension (50 µl) was added to each serum dilution. Dispensing was started at the greatest serum dilution and then worked back, to avoid any risk of serum cross-contamination.

In addition, tenfold serial dilutions of the stock virus were carried out on the plate in order to set up a back titration to check the viral titre.

The virus-serum mixture was held for 90 minutes at room temperature to allow adsorption of antibody to the viral antigen.

MDBK cell cultures were trypsinised and the cells counted [this was facilitated if the monolayers had only recently been passaged and had not reached full confluence]. Cells were diluted in GM to 2×10^5 per ml, and 100 µl aliquots added to each well. To avoid cross-contamination of virus, cells were added to controls first, then from highest dilutions of sera back, then the back titration.

The plates were covered and incubated at $37\ ^0$C in a humidified atmosphere of 5 % CO_2 for 5 days.

Plates were read from the third through to the fifth days. Day four was optimum, as CPE was complete by then, and cell cultures were confluent, not overcrowded, and nutrient levels satisfactory.

Readings were only considered valid if the controls were negative. There was a serum control, which included each test serum plus cells, but without virus, to ensure the serum was not toxic to the cell culture. There was also a cell control, which included only cells diluted in media, and no virus, to ensure that the cells were satisfactory.

Only those plates where the virus titre was confirmed by the back titration were considered valid (dilutions >1 $TCID_{50}$ needed to be positive and dilutions < 1 $TCID_{50}$ needed to be negative, while the wells at 1 $TCID_{50}$ were accepted as positive or negative, as this dilution represents only one virus particle).

As a system control, known positive and negative serum samples were tested periodically during the experiment.

The titre of each serum tested was taken as the reciprocal of the highest dilution which inhibited the development of CPE. Duplicate samples were used for each serum tested, and the inhibition of CPE in one of these wells (or both) was taken as the titre. Sera which did not inhibit CPE at the initial dilution of 1:2 were considered negative. For graphic representation, titres were expressed as Log_2 over time.

All viral work was carried out in a special virus hood (Biological Safety Cabinet Class II, Email Westinghouse Pty Ltd, N.S.W., Australia). The virus hood was tested annually by Total Care Ltd, Onehunga, Auckland.

CHAPTER THREE

INVESTIGATION OF A FIELD OUTBREAK.

3.1 INTRODUCTION

High mortality rates from BVDV infection are only seen rarely and are mostly associated with the Type 2 strain which is not known to exist in New Zealand. Deaths from mucosal disease (MD) are seen sporadically in New Zealand cattle herds, often involving just a single animal. This is because the prevalence of persistently infected (PI) animals in infected herds does not normally exceed 2% (see Chapter 1.7.2), and because it is through superinfection of these PI animals with the cytopathic biotype that MD occurs (Brownlie, 1990). Consequently, if a large number of cases of MD occur in a herd, it implies that a large number of calves must have been born persistently infected at about the same time.

Three episodes have been reported in New Zealand in which high numbers of MD cases were found. The first two of these cases were outlined by Horner (1992) and the third by Frazer (1997).

(i) The first case occurred in 1990, when 40 calves were purchased for beef production. Many became ill-thrifty and unresponsive to repeated anthelmintic or other treatments. They progressively lost condition and after they reached a year of age began to die. Clinical examination revealed oral and digital erosive lesions suggestive of chronic MD. After nine had died, laboratory testing was carried out using a serum neutralisation test and immunoperoxidase assay (SNT/IP) and it was concluded that 17 of the 40 animals (42.5%) purchased in 1990 were PI carriers. Presumably the dams of these calves had been exposed to BVDV on the farm of origin while at a critical stage of pregnancy.

(ii) The second case occurred in 1990 on a beef farm that had two confirmed cases of MD and a number of unexplained deaths over a two-month period in a mob of rising two-year-old bulls. When tested by SNT/IP the remaining 15 animals in this mob were found to be BVD virus positive and antibody negative. They were assumed to be PI and were culled. The rising two-year-old heifers were in very poor condition, and on

testing, seven of these were also found to be PI. Testing of herd sire bulls and rising one-year-old cattle revealed three more PI animals. If the confirmed cases of MD are included, this meant that 27 of the 209 animals (12.5%) tested in this herd were PI carriers. It was suspected that the BVDV infection was introduced into the herd during mating or early pregnancy in 1988/89. None of the sire bulls was found to be PI, but bulls from another farm had also been used and may have been responsible for the infection.

(iii) The third case has been outlined in Chapter 1.7.8. This beef herd showed all the clinical manifestations of BVDV infection, including the death of 20 of 146 calves (13.7%) from MD. The source of infection was suspected to be dairy heifers introduced to the farm, of which three were identified as PI animals.

In a Canadian beef herd of 652 cows and heifers, 51 calves were found to be PI, with only four of them surviving to one year of age (Taylor *et al.*, 1997).

Investigation of the present case began in 1997, following collaborative discussions between MAF, National Centre for Disease Investigation (NCDI), Wallaceville and the Department of Veterinary Pathology and Public Health, Massey University. A calf mortality rate of 37% was recorded on the farm and a diagnosis of mucosal disease had been made. A farm visit was organised to further investigate the problem and this took place on the 24th April, 1997.

3.2 HISTORY

Since calving started in August 1996, 38 calves in a mob of 102 died on a Taranaki dairy farm. Most deaths occurred at 4-6 months of age, from late December 1996 to the end of February 1997, but the problem continued until May, by which time 49 (48%) of the calves had died.

3.2.1 Clinical signs

The following signs were consistently reported in affected animals by the field veterinarian :-

(i) Encrustations around the muzzle and occasional erosions on the buccal mucosa.

(ii) Coronitis, and interdigital ulceration.

(iii) Diarrhoea, with watery faeces, some containing blood.

(iv) Weight loss in most cases.

All animals that showed clinical signs died.

3.2.2 Pathology

The gross lesions consistently reported by the field veterinarian were :-

(i) Oesophageal erosions.

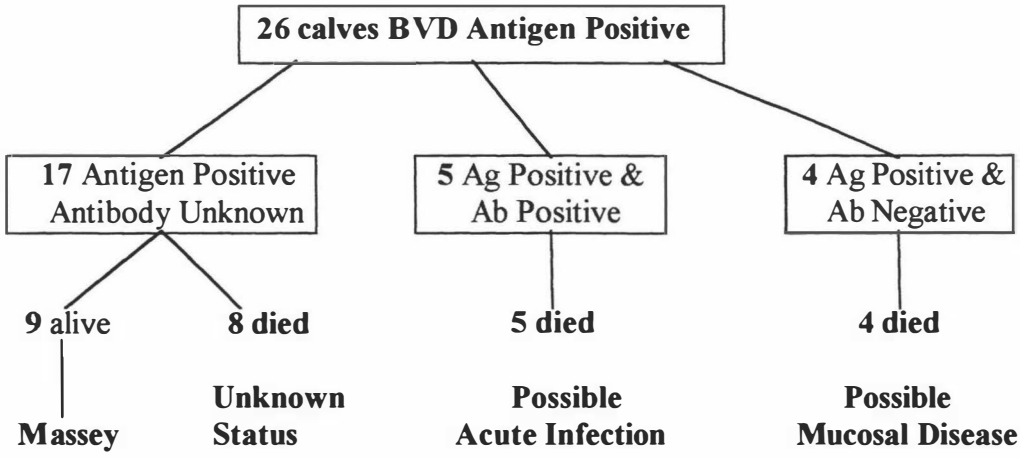
(ii) Red patches in the mucosa of the distal ileum.

The histological lesions in the oesophagus were described as "multifocal erosions in squamous epidermis" and were considered typical of BVD by pathologists at the Batchelor Animal Health Laboratory (BAHL), Palmerston North.

3.2.3 Virology and serology

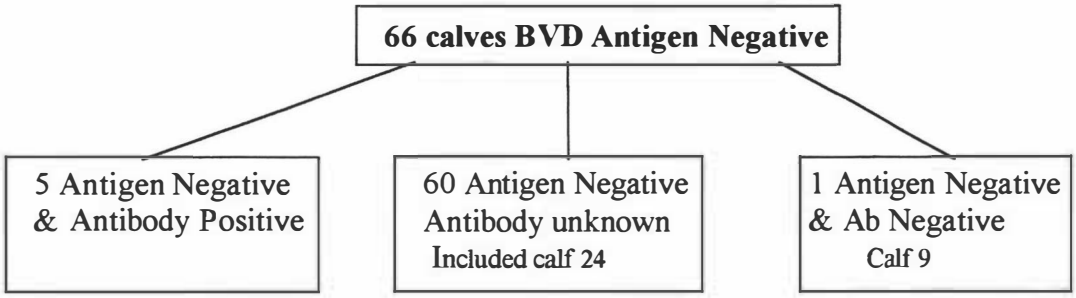
During the period January to March 1997, EDTA bloods were collected from 92 calves in the mob by the field veterinarian. All these were tested for BVD antigen using an ELISA test on buffy coat. Fifteen serum samples had also been collected and were tested for BVD antibody using an ELISA test. These tests were carried out at the Central Animal Health Laboratory (CAHL), Wallaceville. The results of these tests are summarised below :-

A. Calves antigen positive for BVDV.



The nine remaining antigen positive calves were donated to the Department of Veterinary Pathology and Public Health for further investigation. These were identified as numbers:- 12, 15, 26, 28, 39, 43, 66, 67, 96.

B. Calves antigen negative for BVDV



Two of these antigen negative calves were subsequently confirmed positive for BVDV infection. Although calf No. 24 tested Ag negative on the 6th of March (when all remaining calves were tested for antigen) she developed clinical signs of MD and was retested positive for BVD antigen in April. Calf No. 9 had been tested antigen (and antibody) negative, but subsequently developed clinical signs and died. Bovine Viral Diarrhoea Virus (BVDV) was cultured from this animal's spleen at the CAHL.

3.2.4 Diagnosis.

Based on the clinical signs, the tests for both BVDV antigen and antibody and the lesions, a diagnosis of BVDV infection, probably MD, was made. However, it was considered that the possibility of an acute infection by a highly pathogenic strain of BVDV could not be ruled out.

3.3 METHODS AND MATERIALS

3.3.1 General experimental method

Investigations were carried out on the following material :-

(a) Calves submitted to Massey University.

The nine remaining antigen positive calves from the clinical outbreak were blood sampled at the farm (24th April, 1997), transported to Massey University, and placed in a large animal isolation facility. (See Chapter 2.2 for a description of their housing and feeding). Three of the calves were 8 months old, and six were 9 months old. All calves were female. Six calves were Friesians, two were Jerseys, and one a Friesian x Jersey. These calves were clinically examined and blood sampled before euthanasia and necropsy (between the 1st and 9th of May, 1997).

(b) Other samples from farm.

Calf 24 was examined, blood sampled and necropsied on the farm during the initial visit (24th April, 1997). Blood and tissue samples were collected for examination.

Necropsy samples collected by the field veterinarian from four calves (Nos. 61, 79, 85 and 93) were also examined. These were fixed sections of oesophagus and lower ileum that he had collected from calves showing typical erosions in the oesophagus and red patches in the lower ileum.

Two new cases occurred on the farm in early May, 1997 (Nos. 47 and 73). Plain, EDTA, and heparinised bloods were collected by the field veterinarian and submitted from each calf, together with their history. Calf 47 was reported as showing signs consistent with MD, including encrustations around the nose, mouth, eye, neck, coronet and thorax, ulcers at lip commisures and weight loss (12kg in 28 days) but no scouring. Calf 73 showed signs of nasal catarrh, and was provisionally diagnosed as IBR. These blood samples were processed and stored as described in Chapter 2.1.

3.3.2 Antigen

The nine calves submitted to Massey University were the remainder of those that had been tested positive for BVDV antigen (ELISA test) at different times over a three month period (January to March, 1997) by the field veterinarian. At the university BVD antigen ELISA tests were repeated (May '97). Another calf (No. 24) had been blood sampled prior to euthanasia at the farm and this sample was tested for antigen. In addition, blood samples submitted by the field veterinarian (7th May,1997), from two other suspect cases from the farm (calves 47 & 73) were also tested for BVDV. This gave a total of 12 samples to test for BVDV antigen.

3.3.3 Antibody

Serum was collected from ten calves on the farm on 24th April, 1997 (the nine remaining antigen positive calves, plus calf 24) and tested using the ELISA antibody test. These were termed the 1st or "Acute" set of samples. These sera were also tested using the SNT, along with samples collected at necropsy between the 5th and 9th of May, 1997). For convenience these were termed the 2nd or "Convalescent" set of samples (calves 12 and 96, the first two necropsies, were not incuded as it had only been a week since the first samples were collected).

Both ELISA and SNT tests were also used on the two serum samples submitted from the farm (calves No. 47 and 73).

This made a total of 12 calves to be tested for antibody on the first test, and seven on

the second.

The BVD ELISA tests (both antigen and antibody) were carried out in the Central Animal Health Laboratory [CAHL] at Wallaceville, while the SNTs were done in the Veterinary Virology Laboratory at Massey University.

3.3.4 Haematology

Blood samples collected at the university were submitted to the Veterinary Clinical Pathology Laboratory for haematological examination.

3.3.5 Necropsy

Post-mortem examination was carried out on 10 calves, one on the farm (No. 24) and nine at Massey University.

Samples collected at necropsy were processed for histopathology and virology as described in Chapter 2.5.

3.3.6 Virus isolation

Virus isolation was carried out using buffy coat samples and bovine embryonic lung (Belu) cell culture as described in Chapter 2.6.1 and 2.6.2. Passage five Belu cells in small tissue culture flasks were inoculated with the buffy coat samples from ten calves. These included the nine calves donated to Massey, plus a sample sent from the farm (calf No. 47). Two control samples were also prepared, one having no sample added (negative control) while the other was inoculated with a known cytopathic strain of BVDV (positive control). The strain used was NADL, titrated at $10^{6.2}$ $TCID_{50}$ per ml.

After four days, flasks were examined for evidence of cytopathic effect (CPE), frozen to - 70 ^{0}C, thawed and a second pass made in passage 6 Belu cells. This was read after 5 days and the degree of cytopathic effect was scored :-

- = no CPE ; + = mild ; ++ = moderate ; +++ = severe ; ++++ = extreme (complete destruction of the monolayer).

A third pass was carried out on chamber slides with the aim of using immunoperoxidase (IP) staining to confirm the presence of BVDV. Although low passage Belu cells were used (P6), the cell cultures grew very slowly on the glass slides, taking nine days to reach 90% confluence and it was decided that they would be unsuitable for IP staining. However they were innoculated with supernatant from the second pass to observe for CPE.

Samples of supernatant from the second pass were sent to the CAHL at Wallaceville to test for BVDV antigen using the ELISA test, as was a culture sample from the third pass of calf No. 47.

3.3.7 Statistical analysis

The Kruskat-Wallis analysis of variance (based on the ranking of variables) was applied to histological lesion score data to determine significant differences between two groups of calves (Statistix for Windows).

3.4 RESULTS

3.4.1 Clinical signs

Twelve calves were clinically examined, three on the farm (Nos. 24, 47 and 73), and nine at Massey University, before slaughter.

Clinical examination divided the calves into two groups. Half the calves showed minimal clinical signs. Clinical abnormalities were not detected in calves No. 26, 43, and 67, while calf No. 66 had a nasal discharge only and calf No.15 presented with crusty nares and a nasal discharge. In contrast, the remaining six calves (Nos. 12, 24, 28, 39, 47 and 96) presented with significant or extensive clinical signs. A summary of the clinical signs is given in Table 3.1 and a detailed description of each of these signs follows.

Table 3.1 . Clinical signs

Calf Number	Abnormal cardinal signs	Poor general health	Diarrhoea and/or dehydration	Ocular or nasal discharge	Encrustations and/or erosions	Interdigital dermatitis	Enlarged lymph nodes
12	+	+	+	+	+	+	
24	+	+	+		+		
28			+			+	
39		+					
47		+			+		
96	+	+	+	+	+	+	+
15				+	+		
26							
43							
66				+			
67							
73				+			

(a) Cardinal signs.

Only one calf (No. 24) showed a temperature in excess of the critical level (39.5 °C), being recorded at 40 °C. Another calf (No. 96) was subnormal, at 37.8 °C. Calf 12 showed pallor of the mucous membranes.

(b) Poor general health (anorexia, lethargy, condition loss, etc).

Calf 24 was anorexic, moribund and emaciated. Calf 39 remained separated from the rest of the group. The farmer recorded a 12 kg weight loss within 28 days for calf 47.

Calf 96 was in poor condition, and calf 12 was described as 'slab-sided', having a flat rather than rounded lateral profile.

(c) Diarrhoea and dehydration.

Two calves had a watery scour (Nos. 12 & 28) while two others had loose faeces (Nos. 24 & 96). One of these (No. 96) was severely dehydrated and another (No. 12) moderately dehydrated as judged by loss of skin elasticity.

(d) Ocular and nasal discharges.

Five calves had nasal discharges (Nos. 12, 15, 66, 73 & 96) and one of these showed an ocular discharge (No. 12).

(e) Encrustations and erosions.

Crusty erosive lesions were evident at muco-cutaneous junctions in five calves (Nos. 12, 15, 24, 47 & 96). The sites involved were the muzzle, nares, lips/gums and eyes. Calf 47 also had crusty lesions on the neck and thorax, while calf 24 had crusts on the vulva. In addition, both of these calves had encrustations around the coronary band (coronitis). Small (0.5 mm), discrete erosions were evident in the nares in two calves (Nos. 12 & 96).

(f) Interdigital dermatitis.

Three calves walked gingerly and had erosive interdigital lesions in all four feet (Nos. 12, 28, & 96). In two of these calves a crusty dermatitis extended up the volar pastern region to the accessory digits. The lesions were typical of dermatophilosis.

(g) Lymph node enlargement.

Only one calf had palpably enlarged lymph nodes (No. 96).

3.4.2 Clinical pathology.

Tables 3.2 and 3.3 summarise the results of the haemogram and the white blood cell counts (total and differential). Results were available from nine of the twelve calves.

Table 3.2 Haematology results - *Haemogram*

Calf Number	Hb	Hct [PCV]	RBC	MCV	MCH	MCHC	Plat.s	TP	Fib.
12	139	0.42 H	11.56 H	36 L	12 L	330	635	79	3.0
15	137	0.42 H	10.58 M	40 M	12.9 L	320	384	74	2.0
(24)	nr	nr	nr	nr	nr	nr	nr	nr	nr
26	139	0.40 M	9.17	44	15.1	341 H	359	69	5.0
28	nr	nr	nr	nr	nr	nr	nr	nr	nr
39	140 M	0.41 H	11.93	35 L	11.7 L	338	402	73	5.0
43	145 H	0.43 H	9.64	45	15.0	336	516	75	4.0
(47)	131	0.40 M	10.07 M	40	13.0 L	325	453	75	5.0
66	142 H	0.42 H	9.80	43	14.4 M	334	499	78	10.0 H
67	134	0.38	9.31	42	14.3 L	344 H	550	73	5.0
(73)	108	0.32	8.44	38 L	12.7L	334	579	76	4.0
96	nr	nr	nr	nr	nr	nr	nr	nr	nr
Reference Range	80-140	0.24-0.40	5.0-10.0	40-60	14.4-18.6	260-340	200-600	67-85	2.0-7.0
Units	g/l [%}	l/l	x1012/l	fl [u3]	pg	g/l	x109/l	g/l	g/l

LEGEND

( ) = calves sampled on farm
H = above reference range
M = marginal
L = below reference range
nr = no result

Hb = Haemoglobin
Hct = Haematocrit
Plat.s = Platelets
Fib. = Fibrinogen
T.P. = Total Protein

RBC = Red Blood Cell Count
PCV = Packed Cell Volume
MCV = Mean Corpuscular Volume
MCH = Mean Corpuscular Haemoglobin
MCHC = Mean Corpuscular Haemoglobin Concentration

Table 3.3 Haematology results continued. *White Blood Cells -Total & Differential Counts*.

Calf Number	TOTAL W.B.C. COUNT	Segmented Neutrophils	Band Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
12	13.7 H	6.9 H	0.1 M	6.7	0	0	0
		[50%]	[1%]	[49%]			
15	13.6 H	5.9 H	0	6.8	0.3	0.7	0
		[43%]		]50%]	[2%]	[5%]	
(24)	nr	nr	nr	nr	nr	nr	nr
26	7.9	2.3	0	4.5	0.7 M	0.4	0
		[29%]		[57%]	[9%]	[5%]	
28	nr	nr	nr	nr	nr	nr	nr
39	9.7	3.9	0	5.7	0.1	0	0
		[40%]		[59%]	[1%]		
43	8.7	2.4	0	5.2	0.7 M	0.5	0
		[27%]		[60%]	[8%]	[5%]	
(47)	6.7	2.2	0	4.4	0.1	0.1	0
		[33%]		[65%]	[1%]	[1%]	
66	9.4	2.1	0	6.9 *	0.1	0.3	0.1
		[22%]		[73%]	[1%]	[3%]	[1%]
67	11.0	4.5 M	0	5.8	0.7 M	0	0
		[41%]		[53%]	[6%]		
(73)	13.4 H	3.8	0	9.3 H	0.4	0	0
		[28%]		[69%]	[3%]		
96	nr	nr	nr	nr	nr	nr	nr
Reference Range x 10^9/L	4.0 - 12.0	0.6 - 4.5	0 - 0.1	1.8 - 7.5	0.08 - 0.70	0 - 2	0 - 0.2

LEGEND

( ) =samples collected on farm

[ ] = percentage of total w.b.c. count

* = some lymphocytes appear reactive

H = above reference range
M = marginal
L = below reference range

nr = no result

W.B.C. = White Blood Cell

Two calves (Nos. 43, 66) showed a raised Hb level, and one (No. 39) was at the top of the reference range. One calf (No. 12) had a high red blood cell (RBC) count and two (Nos. 15, 47) were marginally so. The erythrocytes appeared normal in each case. However, the results for MCV, MCH, and MCHC indicated that 8 calves had some degree of abnormality in the size and haemoglobin content of erythrocytes. In particular, 6 calves (Nos. 12, 15, 39, 47, 67, 73) show reduced mean corpuscular haemoglobin (MCH) levels.

Five calves (Nos. 12, 15, 39, 43, 66) showed a raised haematocrit (or packed cell volume), while two (Nos. 26, 47) were at the top of the reference range. Total Protein (TP) levels in the plasma of all calves were within normal limits.

Thrombocyte numbers were normal in the nine calves tested for this index.

Only one calf (No. 66) showed a raised plasma fibrinogen concentration, indicating an acute inflammatory process was taking place (this animal had an acute purulent bronchopneumonia, as well as a terminal pancreatitis).

One third of the animals tested (Nos. 12, 15, 73) showed a total WBC count above the reference range. Differential leucocyte counts revealed that calf 12 had a neutrophilia, with the presence of some immature band forms, indicating a left shift. Calf 15 showed a neutrophilia without the presence of immature forms. Calf 73 showed a lymphocytosis. Calf 66 was within normal limits, but some of the lymphocytes appeared reactive. Monocytes were marginally high in three cases (Nos. 26, 43, & 67).

3.4.3 Pathology.

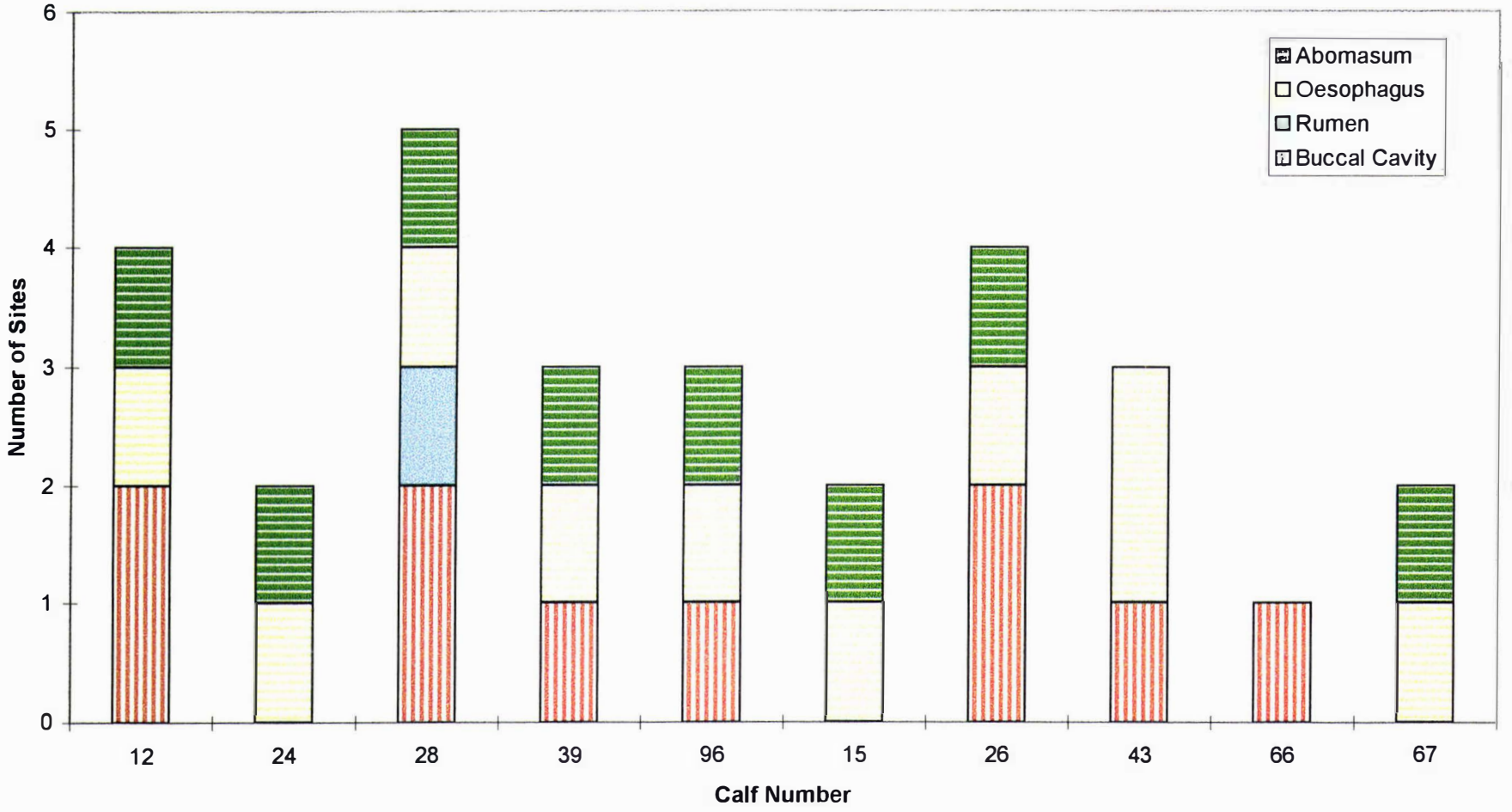
Post mortem examination was carried out on 10 calves, one on the farm and nine at Massey University.

(a) Gross Findings

I Erosions and ulcers of the upper gastro-intestinal tract (GIT).

All calves showed erosive lesions in the upper GIT on necropsy (Fig. 3.1).

Figure 3.1 Distribution of Erosions and/or Ulcers in Upper GIT



The erosions in the mouth of seven calves (Nos. 12, 26, 28, 39, 43, 66 & 96) were found at various sites; gingivae, dental pad, cheeks, hard palate, soft palate, tongue, and pharynx. In most cases they were small (0.5-2 mm diam.) and discrete (Figure 3.2), but in the region of the soft palate they were seen to coalesce into larger erosions with irregular outlines (Figure 3.3). Blunting of buccal papillae was seen in calf 28.

Erosions were present in the oesophagus of all but one calf (No. 66). In five calves (Nos. 12, 15, 24, 26, 67) the erosions were small (1-3 mm diam.), circular and discrete. In five calves (Nos. 12, 28, 39, 43, 96) linear erosions were seen, measuring 2-5 mm long and 1 mm wide. While erosions were mainly seen in the upper third of the oesophagus, in one calf (No. 26) multiple (about 25) 2-3 mm diam. discrete lesions with raised margins were seen throughout the entire length of the oesophagus, while in another calf (No. 12) multiple discrete lesions were also seen along most of the oesophagus, with some longitudinal erosions at the terminal end. In many cases the mucosal surface of the oesophagus had a tattered appearance.

Erosions were seen in the rumen of one calf (No. 28). Two erosions (5 mm and 10 mm diam.) were present on the pillars of the caudo-dorsal sac.

In the abomasum, lesions were seen in 8 of the 10 calves. Multiple pinhead sized (1-2mm diam.) nodules were common in the mucosa, while punctate ulcers up to 5mm in diameter were observed on the sides of rugae, which were also hyperaemic. In one calf (No. 15) abomasal contents contained sanguineous fluid.

In addition to the above, severe lesions of necrobacillosis were seen in the tongue, reticulum, and rumen of calf 24.

All other gross lesions seen at necropsy of affected calves are summarised in Table 3.4.

Table 3.4 Non-erosive gross lesions seen at necropsy of affected calves.

Calf Number	Purulent discharge in URT	Collapse / consolidation in Lung	Enlarged L.N.s	Haem. in L.N.s	Abnormal intestinal contents	Congestion of intestinal mucosa	Enlarged Peyer's Patches	Other findings
12		++	All L.N.s (incl.mes.)		Watery (Jej.) Mucoid (Ileum)	Ileum (plus haem.)		Doudenum. grey & roughened
24		++				Ileo-caecal valve		Grey duodenum Fatty liver
28			S/man.,ileal, prescapular		Watery	Duod., jejunum, ileum		
39					Mucoid	Jejunum (severe)		Friable GIT
96	Nasal cavity		All L.N.s (incl. mes.)		Watery	Ileum, to ileo-caecal valve		Pancreatic haemorrhage
15	Nasal cavity and bronchi		Numerous (incl. mediast.)	++		Distal jejunum & ileum	Raised	
26			Mesenteric			Jejunum	Raised	Enlarged tonsils and thymus
43		+++	Head and mediastinal	++	Mucoid	Duod., jejunum, ileum (mild)		
66	Trachea and bronchi	+			Mucoid	Duod., jejunum, ileum, ceacum		
67					Mucoid	Duodenum, jejunum (severe)		White spot on kidney

Legend :- URT = upper respiratory tract ; LN's = lymph nodes
+ = mild; ++ = moderate; +++ = severe

II Intestinal lesions.

The contents of the small intestine were excessive and watery in three calves (Nos. 12, 28, 96) and mucoid in five calves (Nos. 12, 39, 43, 66, 67). The contents of calf 12 were watery in the jejunum, yet mucoid in the ileum. Congestion was seen at different sites and to varying degrees in the small intestinal mucosa in all cases. The Peyer's patches were raised in two calves (Nos. 15, 26) and the ileo-caecal valve was reddened in one (No. 24). The caecum showed isolated areas of hyperaemia in calf 66, and the GIT of calf 39 was friable throughout its length.

III Lesions in the respiratory tract.

In three calves (Nos. 15, 66, 96) there was purulent discharge in the nasal cavity, trachea and bronchi, whilst in four calves (Nos. 12, 24, 43, 66) there were areas of pulmonary consolidation or collapse.

IV Lymph nodes.

Lymph nodes were enlarged in 6 calves (Nos. 12, 15, 26, 28, 43, 96), and cortical haemorrhages were observed in two (Nos. 15 & 43).

In calves No. 12 and 96, all lymph nodes examined were enlarged.

V Other gross lesions.

Calf 24 had an orange, friable and mottled liver, typical of fatty change.

Calf 67 had a 5 mm diameter white spot on one kidney, which extended 4 mm into the cortex.

One calf had small (1-2 mm) haemorrhages in the pancreas (No. 96) while another (No. 26) had enlarged palatine tonsils and an enlarged thymus (80x 200 mm).

(b) Histopathology

I Lesions in the squamous epithelium of the alimentary tract (mouth to omasum).

A summary of the lesions observed is presented in Table 3.5.

Most calves (80%) showed diffuse parakeratosis of the stratified corneal layer, with the oesophagus the main site affected. When hyperkeratosis and parakeratosis are grouped together, all but one calf was found to have changes in the stratum corneum. Examination of samples of oesophagus collected from four calves necropsied by the field veterinarian (Nos. 61, 79, 85 and 93) revealed moderate irregular parakeratosis in

all cases. Early necrosis of the epithelium, with some neutrophil infiltration, was seen in the oesophagus in one of these calves (No. 79).

Degenerative changes in the epithelium were seen either in the stratum corneum or stratum granulosum. For example, in sections of oesophagus from calf 39, epithelial cells in the stratum granulosum layer showed cloudy degeneration, while in the reticulum, rumen and omasum the stratum corneum layer contained scattered foci of cells showing hydropic change.

Epithelial necrosis was common (60% of calves) and was observed at five different sites. This often appeared to originate in the stratum spinosum (Figure 3.10) before extending to other layers (Figure 3.11) or was superficial, with thickening and desquamation of the stratum corneum (Figure 3.12). Invasion of these necrotic areas by neutrophils and bacteria was common (Figure 3.11).

Cleavage vesicles were observed in the tongue in two cases, as well as in some omasal papillae in one calf (No. 96).

Ulceration was observed in two calves, one in the tongue (No. 26) and the other in the gum and rumen (No.28). Areas of necrosis in the epidermis extended through the basement membrane into the dermis, and neutrophils were present in these lesions (Figure 3.11).

Table 3.5 Histological lesions in the squamous epithelium of the GIT.

Histological Lesion	No. of Calves	Sites involved (number of cases)
Epithelial inflammation	3 (30%)	Oes (1), Ru (3), Ret (3) Om (2)
Epithelial necrosis	6 (60%)	Lip (1), Ton (2), Oes (2), Ru (2), Om (2)
Cleavage vesicles	3 (30%)	Ton (2), Om (1)
Erosions	4 (40%)	Gum (1), Ton (2), Oes (1)
Ulceration	2 (20%)	Ton (1), Gum (1), Ru (1)
Submucosal inflammation	1 (10%)	Ret (1)
Parakeratosis	8 (80%)	Gum (1), Lip (1), Ton (3), Oes (7), Ret (3), Ru (3), Om (2)
Hyperkeratosis	2 (20%)	Oes (1), Ret (1), Ru (1), Om(2)

Legend :-
Ton = tongue
Oes = oesophagus
Ru = rumen
Ret = reticulum
Om = omasum

Although oesophageal erosions had been seen grossly in 90% of calves, only one case was confirmed histologically. The four oesophageal samples submitted by the field veterinarian were from calves with gross erosions, but necrosis of the epithelium was only seen histologically in one calf.

II The abomasum.

Mucosal infiltration by inflammatory cells was seen in four calves (Nos. 43, 15, 67 and 96) with a range of severity from mild to severe. The inflammatory cells were lymphocytes, plasma cells and monocytes. In calf 96 these infiltrations occurred mainly above the lymphocytic nodules.

Epithelial metaplasia was mild in calf 67, with loss of parietal cells only, whereas in calf 15 this change was severe and submucosal oedema was also present. In the epithelium above lymphoid follicles, cells were altered to cuboidal and there was an increased basophilic staining of the cytoplasm. Mitotic figures were increased, and a marked loss of parietal cells was noted.

Moderate focal epithelial necrosis was present in two calves (Nos. 12 & 15) and affected glands were dilated with necrotic debris and neutrophils (Figure 3.9).

Necrosis of mucosal lymphoid follicles was mild to moderate in four calves (Nos. 15, 26, 39 and 67). In calf 39, mucosal lymphoid follicles were moderately enlarged.

Globular leucocytes were seen at the base of the mucosa in three cases (Nos. 15, 39, and 67), especially in the region of the lymphoid follicles. In addition, calf 15 had occasional cross-sections of *Ostertagia* worms in the lumen. In the adjacent mucosa occasional irregularly dilated, hyperplastic glands surrounded by lymphocytes, plasma cells and eosinophils were seen.

III The intestine.

The main histological findings in the intestine are summarised in Table 3.6.

Increased inflammatory cell infiltration of the lamina propria was the most common finding. In all cases the major inflammatory cell was the lymphocyte, while in five cases (Nos. 12, 39, 43, 66 and 67) eosinophils were also present (Figures 3.4 and 3.8). Globular leucocytes were seen in the lamina propria of the duodenum and jejunum in two cases, small numbers in calf 67, and moderate numbers in calf 12.

Destruction of the epithelial lining of the crypts of Lieberkuhn resulting in "crypt abcesses" was only seen in four calves (Nos.12, 24, 28 and 96), but these lesions were severe (Figures 3.4 and 3.5). No crypts were affected in the duodenum, while in

the jejunum lesions were mild to moderate. In the ileum, caecum, colon and rectum lesions were moderate to severe. Crypt dilation was a feature in ileal samples submitted by the field veterinarian (Nos. 61, 79, 85, and 93).

Congestion of mucosal capillaries was seen in the small intestine in 70% of cases, and was most common in the ileum (Figure 3.8). Furthermore, four samples of lower ileum that were submitted by the field veterinarian because they showed "red patches", were found to contain areas of congestion and haemorrhage in the mucosa.

Submucosal haemorrhages in the small intestine were seen in two cases; mild in calf No. 66 and severe in calf No. 24.

Table 3.6. Histopathology of intestinal lesions.

Histological Lesion	No.of Calves	Sites - Scores	Total Score
Congestion of mucosal capillaries	7 (70%)	S.I.(duo.-1; jej.-1, 2, 3; ileum-1, 2, 2, 2, 3)	17
Crypt necrosis of glands of Lieburkuhn	4 (40%)	S.I. - 1, 2, 2, 3, 3 L.I. - 2, 2, 2, 2, 3	22
Inflammation in lamina propria	6 (60%)	S.I. - 2, 3, 3, 3, 4 L.I. - 2, 3, 3	23
Mucosal inflammation over Peyer's patches	1 (10%)	S.I. (jej.) - 2	2
Lysis of Peyers patches (follicular lymphoid tissue)	6 (60%)	1, 1, 1, 2, 3, 4	11
Hemiation of crypts into the submucosa	3 (30%)	S.I. (ileum) - 3, 3 L.I. - 2, 3	11
Submucosal inflammation (eg oedema, haem. etc)	2 (20%)	S.I. - 1, 3	4
Vasculitis and thrombosis	1 (10%)	S.I. - 1	1

Legend :-

S.I. = small intestine; L.I. = large intestine

Score :- 1 = mild; 2 = moderate; 3 = severe; 4 = extreme

Microscopic lesions of Peyer's patches were seen in 6 calves. In three cases there was marked depletion of lymphocytes (Nos. 28, 39 and 96), in two cases there was hyperplasia (Nos. 12 and 26), and in one calf (No. 43) necrosis of Peyer's patches (Figure 3.6). Mucosal inflammation over Peyer's patches was also seen in calf 43. In two samples of ileum submitted by the field veterinarian (Nos. 61 and 85) Peyer's patches were severely atrophic, being filled only by macrophages and occasional small lymphocytes.

Herniation of crypts into the submucosa was seen in the ileum and large intestine in three animals (Nos. 12, 39, 43) and was moderate to severe in extent (Figure 3.7).

Thrombi and fibrin were seen in arterioles in the abomasum and ileum of calf No. 24. Severe necrosis and infarction of arterioles in the lung were also seen in this calf, while another (calf No. 39) showed a mild thrombosis in a pulmonary vessel.

IV Lymphoid tissues.

A. Lymph Nodes

All calves showed lesions in lymph nodes on histological examination and these are listed in Table 3.7.

Table 3.7 Histological lesions in lymph nodes.

Histological Lesion	Calf Identification	No. affected
Congestion	28, 96	2
Oedema	15, 43	2
Haemorrhage	26, 39, 43	3
Lymphocyte depletion	24, 26, 28, 39, 43, 66, 67, 96	8
Necrosis in germinal centres	26, 43, 67, 96	4
Reduced activity (germinal centre)	12, 15, 39, 66, 67, 96	6
Paracortical hyperplasia	12, 15	2

Of the cases showing diminished populations of lymphocytes and necrosis of germinal centres, the mesenteric lymph nodes were the main nodes involved. Depletion of lymphocytes from germinal centres was the most common lesion (Figure 3.14) and was assessed as mild to moderate in severity. Reduced activity in lymph nodes was determined by observation of mitotic figures in germinal centres, as well as through assessment of plasma cell numbers in the medulla. In some lymph nodes where germinal centres were depleted (eg Nos. 67 & 96) a pink amorphous material was present, along with increased numbers of macrophages, giving a "starry sky" appearance (Figure 3.14).

B. Spleen

In calf 28 the spleen was severely congested, and the white pulp areas (periarteriolar sheaths) were depleted of lymphocytes. In calf 43 a small area of necrosis was present in one white pulp area.

C. Tonsils

Lesions were found in the palatine tonsils in calf 26. There was excessive necrotic material in the tonsillar crypts. The lympho-epithelium was very broad, showing as wide eosinophilic zones around the crypts, with mainly mononuclear cells present, as well as neutrophils and bacteria. Focal areas of haemorrhage into the epithelium were also seen.

V The central nervous system.

In the brain of calf 15, there was mild perivascular cuffing by mononuclear cells in the cortex (grey matter) of the posterior cerebrum (Figure 3.15). Some accumulation of mononuclear cells was also seen in the meninges. Mild oedema in the spaces of Virchow was also evident. Perivascular cuffing by mononuclear cells was evident around a few vessels in the posterior cerebrum of calf 28, while in calf 43 there was mild cerebral oedema.

VI The respiratory system

Calf 96 showed a moderate suppurative rhinitis. There was severe congestion of blood vessels in the nasal cavity. Moderate numbers of neutrophils and some eosinophils were present within the nasal epithelium, mostly near the basal layer. Around the submucosal glands, inflammatory cells were also present (neutrophils, eosinophils and some mononuclear cells). This calf had a congenital defect of the head (a twisted 'roman nose') which probably contributed to the URT lesions.

Calf 15 had a severe chronic suppurative rhinitis and also a severe bronchitis. In the primary bronchi, the epithelium showed extensive areas of hyperplasia as well as areas of epithelial necrosis. There was severe generalised submucosal lymphocyte infiltration, with destruction of submucosal glands.

Three calves had a bronchopneumonia. Calf 12 showed collapse and congestion in one lobule of the lung.. Alveoli in this region were filled with fibrin, and bronchi and bronchioles were filled with neutrophils. A few neutrophils were also present in collapsed alveolar spaces. Calf 39 showed a similar picture. Calf 66 had an area of consolidation in the middle right lobe of the lung. The affected bronchi and bronchioles contained necrotic debris, epithelial cells and macrophages. Fibrin was present in many small alveolar blood vessels and within alveolar walls.

VII Other organs.

In the liver, multiple small foci of mononuclear inflammatory cells were present, mainly in the portal triads, of six calves. In five cases these inflammatory foci were mild

Table 3.8a Summary of histological lesion scores in field outbreak calves.

Calf No.	Sq. epith.	Abomasum	Intestine	Vascular	Lymphoid	CNS	Liver	Kidney	Respiratory	Other	TOTAL SCORE
12	16	2	11	2	2	0	1	1	2	0	37
24	10	0	9	12	10	0	2	0	0	0	43
28	2	0	5	0	4	1	1	0	0	0	13
39	21	4	12	1	6	0	1	1	2	0	48
96	12	3	11	0	5	0	0	0	3	0	34
Sub-total	61	9	48	15	27	1	5	2	7	0	175
15	4	12	0	0	3	4	2	0	3	0	28
26	9	2	10	0	5	0	2	1	0	12	41
43	6	1	16	0	7	1	2	1	0	0	34
66	5	0	10	0	3	0	0	1	2	0	21
67	5	5	7	0	4	0	1	2	0	0	24
Sub-total	29	20	43	0	22	5	7	5	5	12	148
TOTAL	90	29	91	15	49	6	12	7	12	12	323

Legend:- Sq. epith. = squamous epithelium of upper alimentary tract
CNS = central nervous system
Red numbers = calves showing extensive clinical signs
Blue numbers = calves showing mild clinical signs

(Nos. 12, 15, 26, 39 and 67), while in one they were moderate (No. 43). The cells were mainly lymphocytes and plasma cells. In one case eosinophils were also present (No. 39).

Other lesions seen were moderate centrilobular fatty change in calf 24, mild fibrosis around bile ducts in calf 28, and mild fibrosis of the liver capsule in calf 67.

Moderate congestion of the renal medulla was seen in half the cases (Nos. 26, 39, 43, 66 and 67). Inflammatory foci of mononuclear cells were seen in the cortex in two calves (Nos. 12 and 67), but these were small interstitial foci located near blood vessels.

In calf 66, the pancreas contained areas of recent haemorrhage, both around blood vessels and within ducts, where some necrotic acinar cells were seen. There was some fibrinous reaction around small blood vessels in the pancreas of calf 96, indicative of a mild vasculitis.

Calf 12 showed lesions in the interdigital cleft. These were very severe hyperkeratosis and parakeratosis, as well as moderate submucosal inflammation (Figure 3.13).

In the adrenal of calf 26 there was an area of moderate congestion in the zona fasciculata.

(c) Summary of pathology

Histological lesion scores are summarised in Table 3.8a. The group of calves showing extensive clinical signs (Nos. 12, 24, 28, 39, and 96) had a total score of 175, while the group with mild signs (Nos. 15, 26, 43, 66 and 67) totalled 148. However, this difference was not found to be statistically significant. Consideration of each organ system revealed that only the vascular lesion scores, which were confined to the group with extensive clinical signs, were significantly different between groups ($p = 0.045$).

Interpretation of the gross and histological lesions have been made, and are summarised in Table 3.8b. The abnormalities found common to all calves were enteritis, erosions, and lymphoid tissue lesions. The types of enteritis encountered were combinations of gastro-enteritis, enteritis and typhlo-colitis. The predominant inflammatory cell seen in the lamina propria of the intestine in all cases was the lymphocyte. Hyperkeratosis and/or parakeratosis was seen in all but one calf.

Table 3.8b Interpretive summary of the gross and histological lesions.

CALF NUMBER	24	28	26	15	43	66	39	67	96	12
Enteritis										
Keratosis										
Erosions										
Abomasitis										
Oesophagitis										
Omasitis										
Rumenitis										
Lymph node lesions										
Pancreatic lesions										
Rhinitis										
Broncho-pneumonia										
Vasculitis										
Thrombosis										
Necrobacillosis										
Tonsilitis										
Meningo-encephalitis										

Figure 3.2 Hard Palate of calf 12. 3x

Numerous erosions are evident on the ridges of the hard palate, as well as on the dental pad.

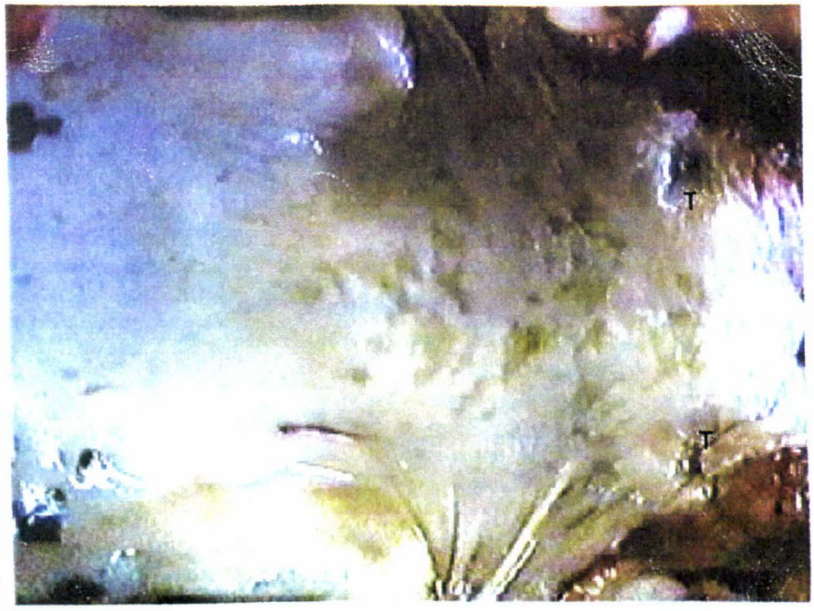


Figure 3.3 Soft palate of calf 12. 3x

The erosions have coalesced in the region of the pharynx, anterior to the palatine tonsils (T).

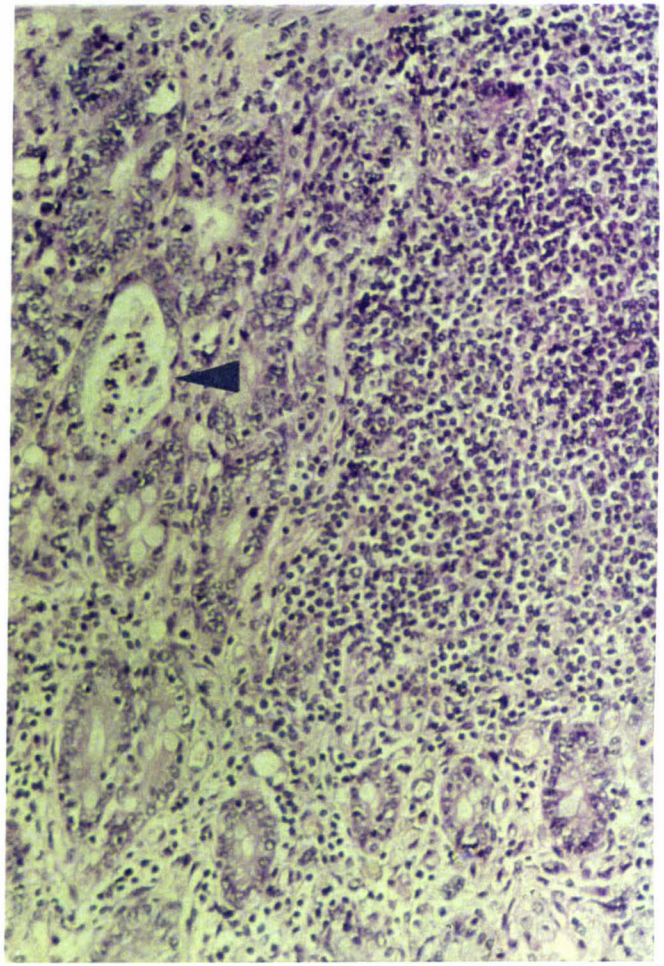


Fig. 3.4 Calf 24, Ileum. Inflammation at the base of the mucosa, with lymphocytes being the major infiltrating cell. One crypt (arrow) contains a number of neutrophils ("crypt abscess"). 150x

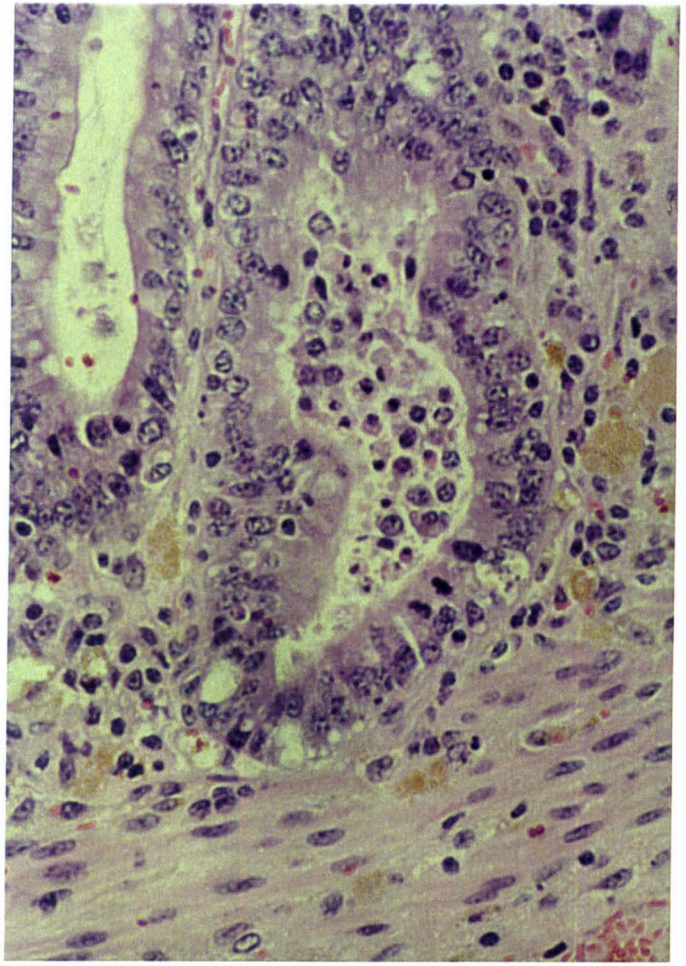


Fig. 3.5 Calf 96, Ileum. A dilated crypt contains neutrophils, many of which are necrotic. Haemosiderin is present in the surrounding tissues. 600x

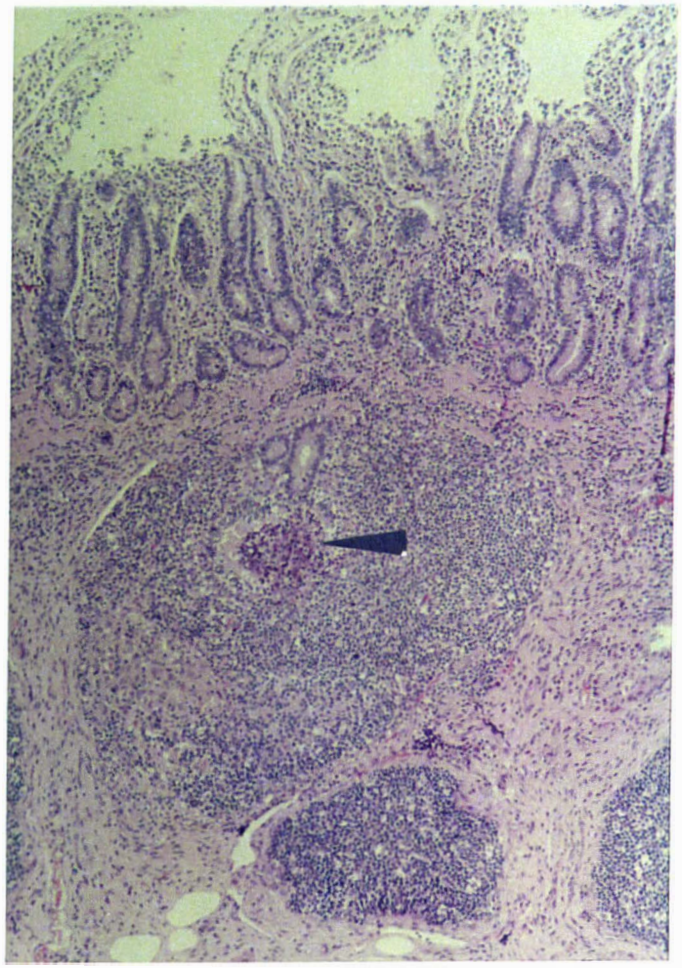


Figure 3.6 Calf 43. Ileum. 150x
A Peyer's patch contains a focal area of necrosis (arrow).

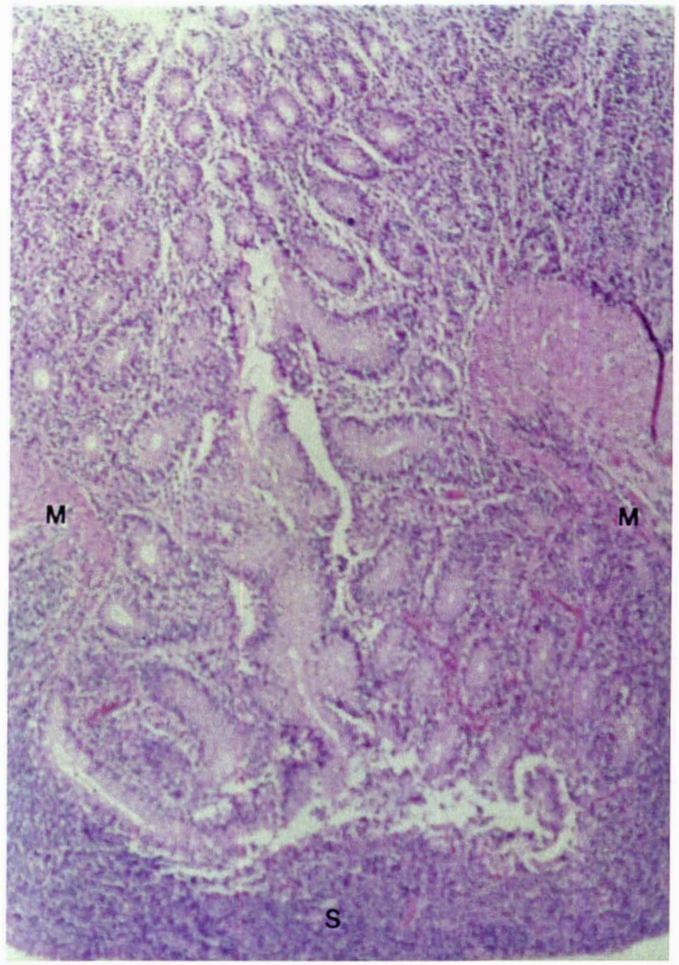


Figure 3.7 Calf 39. Large Intestine. 150x
Crypts are prolapsed through the muscularis mucosa (M) into the submucosa (S).

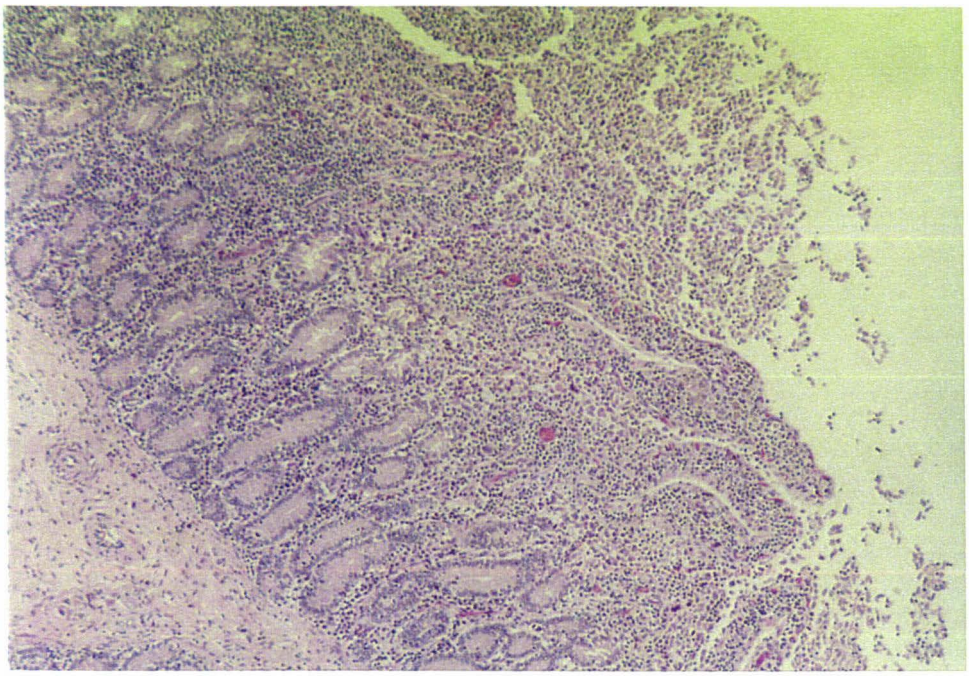


Figure 3.8 Calf 66. Ileum. 150x
Congestion of mucosal capillaries and diffuse lymphocyte infiltration of the lamina propria.

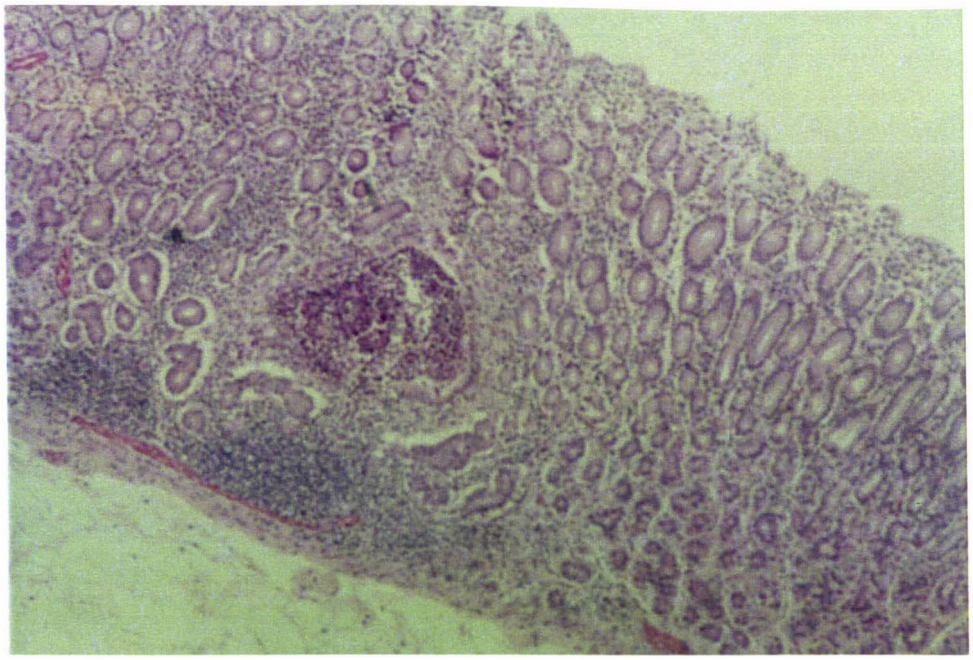


Figure 3.9 Calf 15. Abomasum. 150x
Gland dilation and necrosis, with active lymphoid follicles below it.

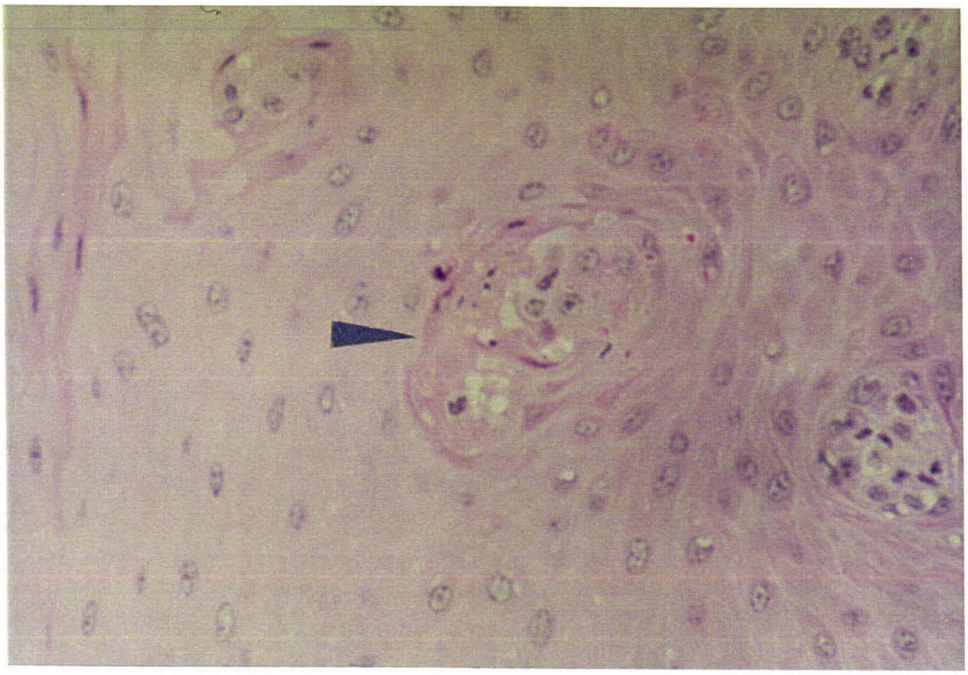


Figure 3.10 Calf 26. Tongue. 600x
A focus of epithelial necrosis in the stratum spinosum of the epidermis.

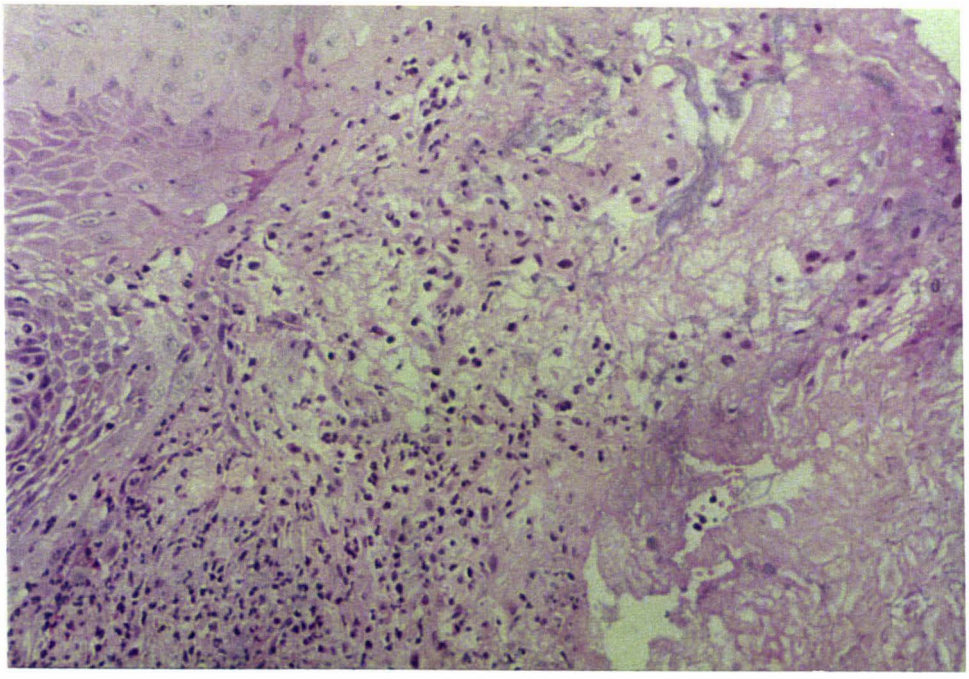


Figure 3.11 Calf 28. Gum Ulcer 150x
There is an area of necrosis in the epidermis, which extends through to the basement membrane. Neutrophils are migrating into the area and bacteria are present (seen as basophilic cords).

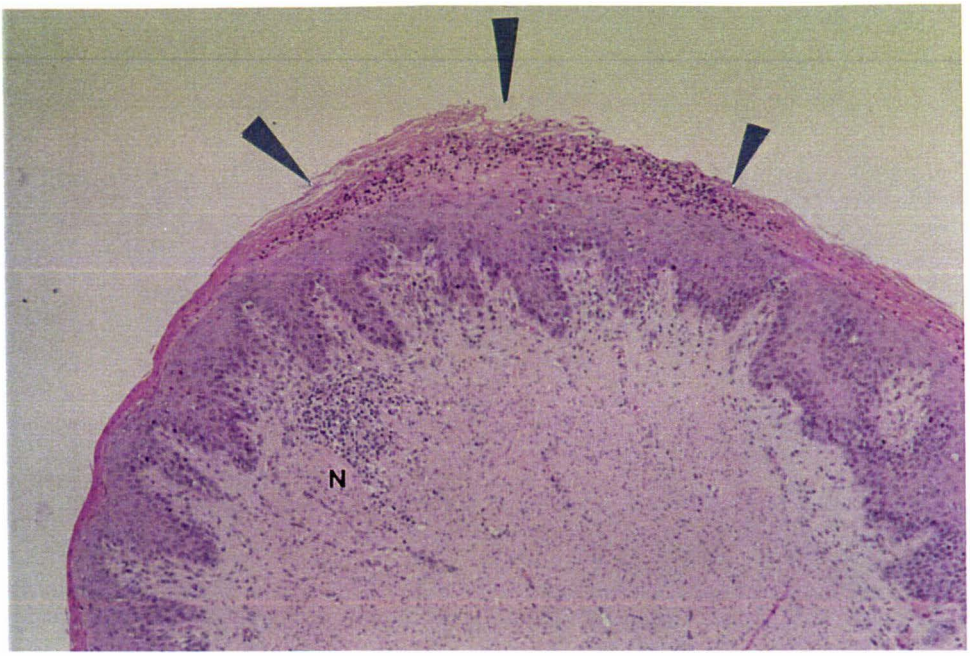


Figure 3.12 Calf 96. Omasum. 60x
Superficial necrosis of the epidermis at the tip of a papillae.
The stratum corneum is thickened and desquamating and shows invasion by neutrophils. A focus of neutrophils is also present in the dermis (N).

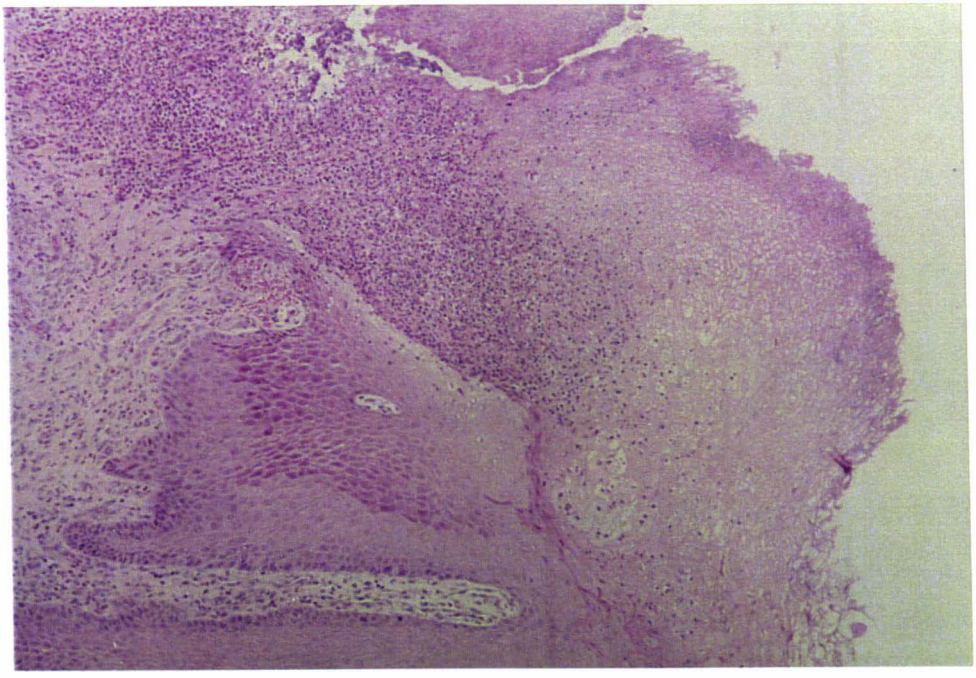


Figure 3.13 Calf 12. Interdigital cleft. 60x
Severe hyperkeratosis and ulceration. The basement membrane has been breached and inflammation (mainly neutrophils) extends into the dermis.

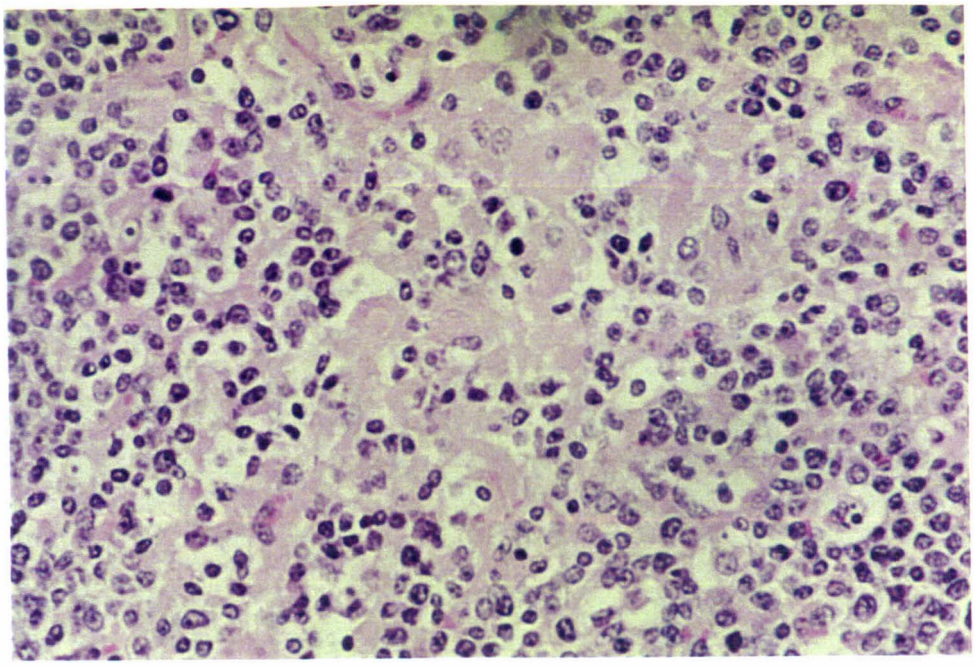


Figure 3.14 Calf 67. Mesenteric Lymph Node. 600x
Depletion of lymphocytes and reduced activity in a germinal centre. Amorphous acidophilic material is seen in the empty spaces.

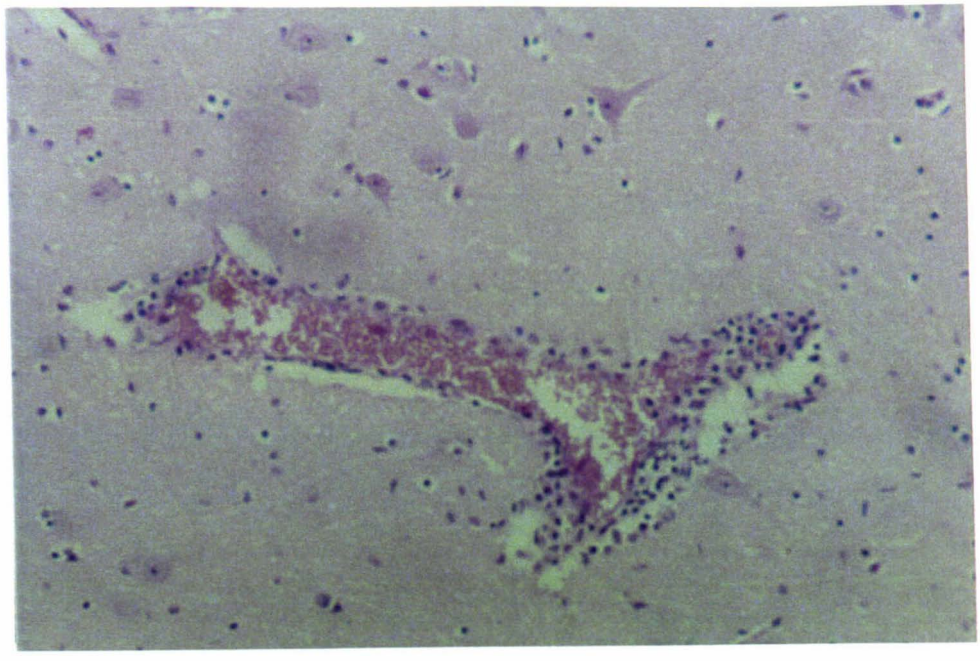


Figure 3.15 Calf 15. Posterior Cerebrum. 300x
Mild perivascular cuffing by mononuclear cells in the cortex.

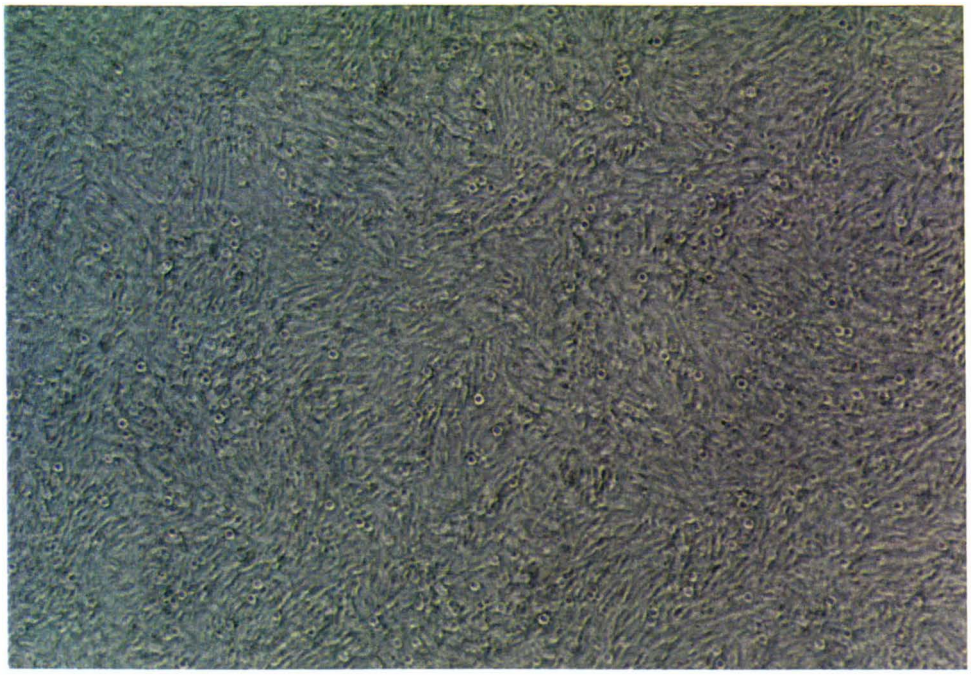


Figure 3.16 Bovine Embryonic Lung (Belu) cell culture.
Control plate. 120x

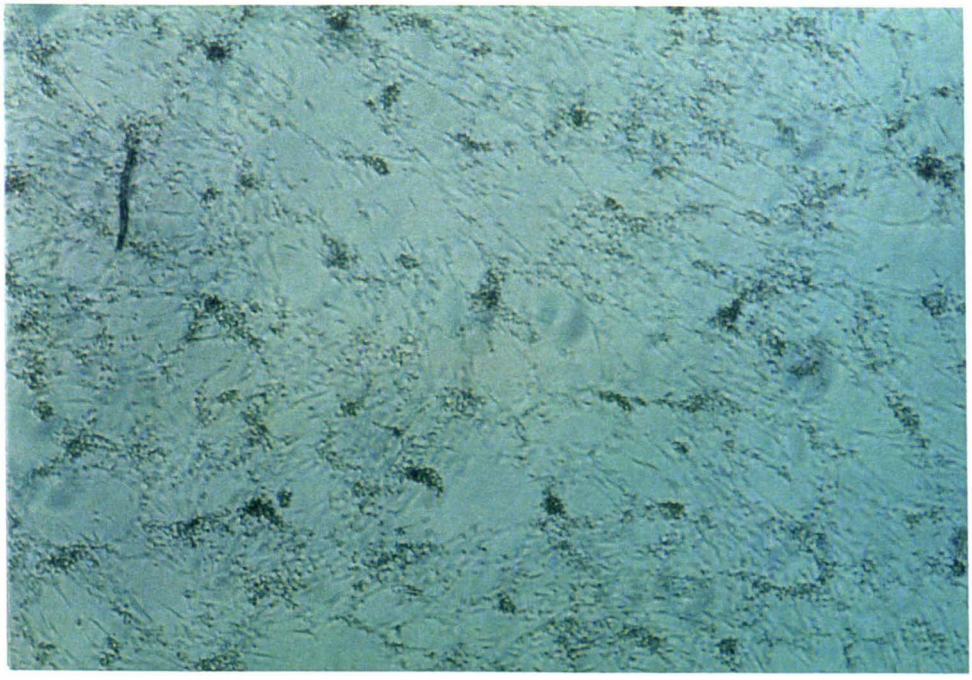


Figure 3.17 Buffy coat sample from calf 12 on Belu cells.
Second pass, showing severe cytopathic effect. 120x

3.4.4 Microbiology results

Mesenteric lymph node and ileal contents were collected from three calves (Nos. 12, 66 and 96). No organisms were seen on direct microscopy, and no growth was observed on culture of the lymph nodes. No *Salmonella* or *Yersinia* were isolated on culture of the intestinal contents.

3.4.5 Antigen and Antibody results

Blood samples from a total of twelve animals were tested for antigen and antibody.
The antigen and antibody results have been tabulated along with the virology results in Table 3.9.

The BVD antigen test carried out while at Massey University was found to divide the calves into two groups, six being antigen positive (Nos. 12, 24, 28, 39, 47, 96) and six being antigen negative (Nos. 15, 26, 43, 66, 67, 73).

A. *The BVD antigen positive group* were the animals that had shown more severe clinical signs, which were indicative of mucosal disease. Although the BVD antibody ELISA tests showed variable results, the SNT revealed that these calves all had low (<16) or negative antibody titres.
Further results from calves in this group include:-
(i)Despite testing negative for antibody by the ELISA test in the first ("acute") sample, calves 28 and 39 showed low titres of 8 and 2 in the SNT. The second ("convalescent") blood samples collected from these two calves showed a minor drop in antibody titre to 4 and 0 respectively in the SNT.
(ii)Calf 24 showed CPE in its serum control wells during SNT testing, despite all other sera controls being negative. This was repeated with the same result at a later date. [Note that all sera were heat inactivated].
(iii)Calf 47 was positive for both antigen and antibody, with an SN titre of 16.

Table 3.9 Antigen, Antibody and Virology Results.

	ANTIGEN TESTS		ANTIBODY TESTS			VIRUS ISOLATION	
CALF	ELISA		ELISA	SNT Titres		CPE	Ag ELISA[a]
ID	Farm	Massey University	1st or "Acute"		2nd or "Conv."	Buffy Coat	2nd Pass
	Jan - Mar ' 97	April - May ' 97	April ' 97		May '97	June ' 97	Nov ' 97
12 #	Positive	Positive	**Positive**	4		**Positive +++**	**Suspicious**
(24) #	Negative	Positive	Negative	*		----	----
28 #	Positive	Positive	Negative	8	4	**Positive ++**	Negative
39 #	Positive	Positive	Negative	2	0	**Positive ++**	**Positive**
(47) #		Positive	**Positive**	16		Negative	**Positive**
96 #	Positive	Positive	Negative	0		**Positive +++**	**Positive**
15	Positive	Negative	**Positive**	256	512	Negative	Negative
26	Positive	Negative	**Positive**	2048	4096	Negative	Negative
43	Positive	Negative	**Positive**	2048	2048	Negative	Negative
66	Positive	Negative	**Positive**	512	512	Negative	Negative
67	Positive	Negative	**Positive**	2048	2048	Negative	Negative
(73)^		Negative	**Positive**	1280		----	----

Legend # = showing clinical signs of mucosal disease.
( ) = sample only submitted to Massey
^ = suspect IBR case.

* = serum control positive
++ = moderate ; +++ = severe
[a] = test conducted on supernatant from second pass in Belu cells.

B. *The BVD antigen negative group* were the animals showing mild clinical signs.
Of the nine calves previously found antigen positive by the field veterinarian, five of these were now BVD antigen negative.
All calves in the BVD antigen negative group were antibody positive to the ELISA test, and showed high antibody titres (>256) to the SNT in the first ('acute") samples.
Further results from calves in this group include:-
(i)"Convalescent" samples from calves 15 and 26 showed rises in antibody titres in the SNT (from 256 to 512 and 2048 to 4096, respectively).
(ii)One sample submitted from the farm (No. 73) was a suspected Infectious Bovine Rhinotracheitis (IBR) case, and this animal showed a high antibody titre of 1280 in the SNT.

3.4.6 Virus isolation results

Virus isolation was carried out using buffy coat samples collected from the nine calves donated to Massey, plus a sample sent from the farm (calf 47). The virus isolation results are summarised in Table 3.9 along with the antigen and antibody results.

Four days after Belu cells were inoculated with these samples, there was evidence of cytopathic effect (CPE) in some culture flasks, including the positive control, while the negative control had a healthy monolayer. A second pass was carried out, and this was read after 5 days. CPE of varying degrees was observed in cultures from calves No. 12, 28, 39 & 96 and was not observed in cultures from any other calves. The degree of CPE was moderate in calves No.28 and 39, and severe in calves No. 12 and 96. There was no CPE in the negative control, and extreme CPE in the positive control, with complete destruction of the monolayer. Figure 3.16 illustrates the negative control sample and Figure 3.17 the Belu cell culture of calf 12 showing severe CPE.
Samples of supernatant from the second pass sent to the CAHL at Wallaceville revealed that BVDV antigen was present in cultures from calves No. 39, 47 & 96. A suspicious result was obtained for culture of calf No. 12, and all other cultures were negative.
Considering the BVD antigen positive group described in the previous section, with the exception of calf 47, CPE was observed in these animals and this was confirmed to be due to BVDV in all but one calf (No. 28). The calves in the BVD antigen negative group were all negative for virus isolation.

A third pass was carried out, and CPE results were the same as at the second pass. In particular, calf 47 was negative for CPE in tissue culture after three passes, yet remained positive for the presence of BVDV antigen.

3.5 DISCUSSION

The pathogenesis of BVDV has been reviewed by Brownlie (1990) who described a wide range of severity for bovine pestivirus disease, from 'acute' infections, which may be inapparent or mild, to mucosal disease which is invariably fatal. The severity of the present episode, with high morbidity and mortality of calves, is unusual for the disease in New Zealand and raised the possibility that a previously unrecognised highly virulent strain of the bovine pestivirus was involved. The serology and virology results have supported the accepted pathogenesis of the disease as described by Brownlie. The two biotypes of the virus, non-cytopathic (NCP) and cytopathic (CP), have a sequential role in the pathogenesis of mucosal disease. Initial foetal infection by the non-cytopathic virus, before the immune system has developed, may result in the production of persistently viraemic calves. These calves are specifically immunotolerant, and do not produce high levels of antibody despite generalised viral infection. They may later develop mucosal disease as a result of superinfection with an antigenically homologous but cytopathic strain of BVDV. The classical laboratory features of mucosal disease are, therefore, a positive test result for the presence of virus or viral antigen, with a negative result for specific antibody. Isolated virus is typically cytopathic in tissue culture.

In this study, the six calves which remained antigen positive had either negative or low antibody titres. In two cases (calves No. 28 and 39), convalescent samples were taken 11 and 13 days later, respectively. Despite this small time interval, the titres had fallen, which may indicate that these titres represented low levels of declining colostral (maternal) antibody. However, as the titres varied only by one two-fold dilution, this difference could merely represent between-test variation. Another explanation for the presence of antibody in persistently infected calves is given by Penny et al, 1996. They proposed that foetal infection at a time when the immune system is starting to develop (around 100-150 days of gestation) may still result in persistently infected calves, but that these calves would also be able to produce small amounts of antibody. This seems rather unlikely, as it could be expected that any antibody produced would combine with

antigen and no longer be detectable.

Four of the five antigen positive calves yeilded virus that showed CPE in tissue culture, which confirmed the presence of the cytopathic biotype, a prerequisite for mucosal disease. The calf not showing CPE (No. 47) also had an antibody titre of 16, so was diagnosed as either persistently infected with the NCP biotype, or an early acute infection with NCP virus, where the titre was starting to rise. Clinically, however, this calf was reported to be showing signs suggestive of chronic mucosal disease. There was a history of weight loss without scouring, crusty erosive lesions at muco-cutaneous junctions, and crusty lesions in the neck, coronet and thorax. These lesions are unlikely be the result of long term persistent infection. In addition, the possibility that this was an acute infection by a highly virulent strain was not likely, but could not be eliminated. The isolate from calf 47 was chosen for use in the transmission experiment to help clarify this picture, and also because it was a NCP biotype (Chapter 4).

One antigen positive calf (No. 24) was *in extremis* on the farm and showed severe signs of mucosal disease. The serum sample from this animal was negative for virus-neutralising antibody. However, the serum controls showed CPE, while the controls for other calves were all negative. This result was repeated when the same sample was tested at a later date. Although it is possible that the serum was toxic to cells at high concentration, another explanation for this result could be that this calf was viraemic at the time of blood collection, with CP virus being present in the peripheral blood. The heat inactivation process performed on all sera used for SNTs was expected to inactivate any virus that was present. Taylor et al (1963) found that the BVD virus was rapidly inactivated at 56^0C in one hour. The same temperature was used in this study, but only for 30 min., so if the titre of virus was very high, then perhaps sufficient virus survived this treatment.

In contrast to mucosal disease, simple acute BVDV infections are common. In New Zealand, about 60% of adult cattle, whether dairy or beef, have seroconverted to BVDV (Golo, 1989; Perez et al, 1994; Perez et al, 1995). The BVDV isolated from acute infection is NCP, and this biotype is the one normally circulating within cattle populations (Brownlie 1990). The laboratory features of acute infection are a negative test result for viral antigen after about 10 days, with a positive test result for specific

antibody after about 10 days with a slow rise to a maximum titre at 10-12 weeks. Virus isolated within 10 days of infection does not produce CPE in tissue culture (Brownlie, 1990).

In this study, all five calves that were initially antigen positive on the farm and then were subsequently found negative for antigen after arrival at Massey University, fit the above criteria, having high antibody titres at least two months later, and being negative for virus isolation. In addition, two of these calves (Nos. 15 & 26) demonstrated a rise in antibody titre in convalscent samples collected 15 and 13 days, respectively, after the first (acute) samples. The fact that the time interval between samples was narrow may make this finding significant. Conversely, this degree of change could also be explained merely by between-test variability.

It is noteworthy that two calves (Nos. 28 and 39) that were negative to the ELISA test for antibody, showed low titres in the SNT. This could mean that the SNT is simply more sensitive than the ELISA test. Alternatively, this could also relate to the fact that the two tests detect antibody to different viral antigens (Horner, 1996). The SNT detects antibody to an envelope glycoprotein associated with virus neutralisation [gp53] whilst the ELISA measures a non-structural protein produced by replicating virus [p125/80].

Four possible false negative BVD antigen ELISA tests were found during this study. These animals developed signs of the disease, were tested negative for BVD antigen, and susequently died. Two of these cases (Nos. 9 and 24) were reported in Section 3.2 and both were confirmed positive for BVD antigen in subsequent tests (virus isolation from spleen and BVD antigen from buffy coat, respectively). On the farm, one further death was reported after the nine calves were donated to Massey University. This was a calf which previously tested BVD antigen negative, but developed signs consistent with BVDV infection and died. Fourthly, calf No. 28 that was positive for CPE, yet negative for BVDV antigen in culture, showed pathology suggestive of BVDV infection.
A total of 114 BVD antigen ELISA tests were carried out during the outbreak, 92 while on the farm and 22 while at Massey University. A total of 36 BVD antigen ELISA tests (26 on the farm and 10 at the university) were positive, and all these calves developed BVD. Of a total of 78 negative BVD antigen ELISA tests (66 on the farm and 12 at the university), 74 of these calves were shown to be truly negative (did not develop BVD),

while the four calves described above did develop disease (Table 3.10).

Table 3.10 Antigen test vs Disease status.

	BVD +ve	BVD -ve	Total
ELISA +ve	36	0	36
ELISA -ve	4	74	78
Total	40	74	114

Calculations from this data reveal that the sensitivity of the BVD antigen ELISA test used in this study was 90% and the specificity was 100%. The positive predictive value was 1 and the negative predictive value 0.95. It could therefore be concluded that when testing for BVD antigen using the CAHL, most animals testing positive to this ELISA will really have the disease, whereas there is a 5% chance that those testing negative may actually have bovine pestivirus disease. However, these calculations have been made with data from cattle in one herd and in the midst of a large outbreak of BVDV associated disease. Actual values for sensitivity and specificity may be different if larger numbers of animals are tested, particularly in different locations.

When the clinical signs are considered in the light of the blood test results, it can be seen that it is the antigen positive calves which showed the most severe clinical disease, while the antigen negative calves showed only mild signs (Table 3.1). The clinical signs seen in the antigen positive calves were those typical of mucosal disease, with some signs being suggestive of chronic infection. eg chronic erosive lesions (encrustations) at the junction of skin with mucous membranes or lesions at skin to horn junctions (coronitis and interdigital ulceration). The chronic lesions seen in calf 47 suggested that it was PI animal, rather than one suffering an early acute infection.

The main haematological abnormality was seen in the haematocrit values, with five calves showing a raised haematocrit, while two were at the top of the reference range. The haematocrit is considered the most accurate determination of an animal's haemogram (Benjamin, 1961), with error being low (in the order of 1-2%). Normal values can vary considerably according to breed, age, activity and altitude. The dairy breeds are considered to have a lower limit (35%) than the beef breeds (40%) [Jones et al, 1956]. Applying these ranges to the present data would mean that all but one calf

would be in the high catagory, indicating that dehydration is a common sequel to bovine pestivirus infection. However, total protein levels in the plasma of all calves were within normal limits, which suggests that the dehydration indicated above was not extreme.

There was some degree of abnormality in the size and haemoglobin content of the erythrocytes in 90% of the calves. This probably indicates that BVDV infection was impinging on erythrocyte function in some way. This could possibly relate to the recently proposed effect of the virus on bone marrow (Marshall *et al.*, 1996).

At necropsy, all calves examined showed lesions typical of BVDV infection, irrespective of their diagnosed status (based on antigen, antibody and virology tests). Three lesions were common to all calves; enteritis, erosions (and/or ulcers) and lesions in lymphoid tissues. In New Zealand, this combination of lesions is strongly indicative of bovine pestivirus disease, as few other diseases have these lesions. The antigen positive calves had more erosive lesions in the upper alimentary tract, than the antigen negative calves. Parakeratosis and/or hyperkeratosis was also widespread and apparently unrelated to disease status. Vascular lesions were only seen in mucosal disease cases, as was omasitis and rumenitis, but the numbers involved were small. Crypt necrosis of glands of Lieberkuhn was only observed in calves diagnosed as mucosal disease. However, this lesion has recently been reported in an experimentally infected calf using NCP virus, New York-1 strain (Marshall *et al.*, 1998).

The value of necropsy in the diagnosis of BVDV infection was clearly demonstrated in this study. On clinical examination only one calf was observed with enlarged lymph nodes, and only four were found with erosive lesions of the head and mouth. However, following necropsy, lymph node enlargement was confirmed in six calves, with two of these calves showing enlargement of every lymph node examined. In addition, erosions and/or ulcers were observed in the upper alimentary tract in all ten calves. Although oesophageal erosions were seen grossly in 90% of calves, only one was confirmed histologically. Similarly, the four oesophageal samples submitted from the field veterinarian from animals with visible erosions could not be confirmed histologically. This may reflect the difficulty of trimming in these small and superficial erosions, rather than any misdiagnosis. It also demonstrates the importance of gross anatomical

pathology in recognising this lesion. Careful examination was required, however, as superficial erosions were sometimes rather inconsequential and easily overlooked.
The value of careful investigation of the Peyer's patches during necropsy was also highlighted by this study, as both their gross and microscopic appearance is important in the diagnosis of BVDV infection. The finding of deep red-black Peyer's patches (visible through the serosa) is a distinctive lesion that is paralleled only in rinderpest, a highly contagious disease of cattle characterised by erosive or haemorrhagic lesions of all mucous membranes (Barker *et al.*, 1993).

The microbiology results from three of the calves that were scouring confirm that the enteritis seen was not due to bacterial infection, namely *Salmonella* or *Yersinia* species.

Histological examination of the intestines demonstrated a lymphocytic enteritis in all cases. A search for the presence of crypt "abscesses" and/or herniation of crypts into the submucosa was particularly valuable, as these lesions were very distinctive and are deemed to be characteristic of bovine pestivirus disease (Barker *et al.*, 1993). There is destruction of the epithelial lining of the crypts, which become dilated and filled with mucous, epithelial debris and leucocytes. This gives them a distinctive abscess-like appearance in the lower intestinal mucosa. Later in the course of the disease these dilated crypts can herniate into the submucosa, replacing involuted lymphoid follicles in Peyer's patches (Barker *et al.*, 1993).

The two cases of meningo-encephalitis and the vascular lesions observed confirm that in some calves it may be difficult to differentiate BVDV from malignant catarrhal fever (MCF). A reported case of BVDV in a New Zealand stud Hereford herd was initially misdiagnosed as MCF (McNeill and Van der Oord, 1993).

Congestion of the mucosal capillaries was seen in the small intestine, especially in the ileum. This was likely to be related to the method of euthanasia used (intravenous barbiturate). However, mucosal congestion and haemorrhage was also seen in samples submitted by the field veterinarian and these were from specific sites of reddening seen grossly in the lower ileum in calves that had died naturally. Further study is needed to determine whether this is a significant lesion in BVDV infection.

Concurrent infection with gastrointestinal parasites was indicated in seven of the ten cases examined post mortem.

In the abomasum, the presence of multifocal, small (1-2 mm) nodular lesions were considered to be of parasitic origin (*Ostertagia spp*) while the punctate ulcers (up to 5 mm) on the sides of rugae were considered to be BVDV related. This was supported by subsequent histological findings, including the presence of globular leucocytes in the lamina propria of the abomasum. However, some of the microscopic lesions seen could well have been attributed to parasites rather than BVDV infection. For example, in calf 15, the area of focal necrosis in a Peyer's patch (Fig. 3.6) or the gland dilation and necrosis seen in the abomasum (Fig. 3.9) could well be due to invading parasitic larvae.

In the small intestine, the presence of eosinophils in the lamina propria of five calves probably indicated that a hypersensitive reaction was taking place. This was probably the a result of nematode parasitism. Globular leucocytes were also present in two of these five calves. In these cases it was difficult to determine whether the marked inflammatory response was due to BVDV or nematode parasite antigen.

The role of BVDV as an immunosuppressive agent is demonstrated in the high frequency of respiratory disease in five of the ten cases. Severe lesions were seen in the upper respiratory tract in calf 15 and it was suspected that a combined viral infection could be taking place (eg with IBR, PI3, or BRS). One sample submitted from a calf with suspected IBR showed a high antibody titre to BVD. It is possible that this calf had a mixed BVDV and IBRV infection. A serological study carried out in New Zealand by Motha *et al.* (1997) to determine the viral aetiologies for bovine respiratory disease indicated that mixed viral infections do occur. However, a nasal discharge is a common feature in early BVDV infection (around 3-7 days) and this can be clinically similar to IBR (Anderson PD, unpublished).

The proposed pathogenesis for this outbreak is as follows. Initially the herd may have been naive for BVDV, and analyses by the field veterinarian supported this (eg deaths of antigen positive calves occurred in all age groups). Infection could have occurred when the farmer introduced six bulls into the herd after five weeks of artificial breeding. Infection around this time of gestation (45-90 days) could have resulted in the production of a large number of PI calves. Subsequent infection with the cytopathic

biotype would have then resulted in mucosal disease. Infection of the cows may have resulted from a PI bull, as antibody testing of 20 cows, carried out by the field veterinarian, revealed that they had all been exposed to BVDV, indicating a widespread infection. The six bulls were all slaughtered after mating, so this hypothesis could not be confirmed.

Although this severe outbreak of bovine pestivirus disease was consistent with the introduction of BVDV infection in a naive herd, the possibility that a highly virulent strain of the virus was involved could not be ruled out, and further investigation was deemed necessary, such as characterisation of the virus by both *in vivo* and *in vitro* testing.

3.6 SUMMARY.

I. High mortality was a feature in an outbreak of confirmed bovine pestivirus disease in a mob of replacement heifer calves in a dairy herd.

II. Immunological and virological studies of twelve calves towards the end of the outbreak revealed that six were acute BVDV infections, five were mucosal disease, and one was infected with the NCP biotype only.

III. A detailed clinical and pathological investigation of the calves allowed a comparison of the signs and lesions associated with "acute infection" versus "mucosal disease". It was found that although clinical signs were more widespread with mucosal disease, pathological lesions were common to both groups, and differences in degree were not found to be significant.

IV. Three lesions were found to be common to all calves - enteritis, erosions (and/or ulcers) and lymphoid tissue lesions.

V. The finding of crypt necrosis of the glands of Lieberkuhn and herniation of crypts into the submucosa were two distinctive microscopic lesions that may be diagnostic for bovine pestiviral disease in New Zealand.

VI. The importance of a careful post mortem examination in the diagnosis of bovine pestivirus disease was demonstrated.

VII. It is possible that BVDV infection was a contributing factor in the establishment of other diseases such as parasitic gastroenteritis (70% of cases), respiratory disease (50% of cases), dermatophilosis (2 calves) and necrobacillosis (1 calf).

IX. The outbreak was diagnosed as a severe case of mucosal disease, starting with a large number of PI calves resulting from BVDV infection of a naive herd between 45 - 125 days of pregnancy, followed by the introduction of a cytopathic biotype (possibly a mutant of the NCP biotype).

X The possibility that a highly pathogenic strain of BVDV was involved in the outbreak was unable to be ruled out, as widespread and severe pathology was found in the acute cases, the symptoms of one calf (No.47) did not fit its diagnosed status, and all calves that developed clinical signs on the farm eventually died.

CHAPTER FOUR

EXPERIMENTAL TRANSMISSION OF A PESTIVIRUS.

4.1 INTRODUCTION

The pathological consequences of experimental infections with NCP isolates of BVDV are poorly documented (Marshall *et al.*, 1998). The first reported experimental infection was carried out in calves using an isolate recovered from a sick cow in 1948 in New York State (strain NewYork-1). Infection with this Type1 BVDV resulted in a diphasic febrile response, leucopenia, general malaise and diarrhoea in some animals (Baker *et al.*, 1954). Two of 105 calves experimentally infected died, and oral ulcers were the only lesions seen grossly and microscopically. In two other studies using the New York-1 strain, the clinical features of experimental disease were reported, but the lesions were not described (Ellis *et al.*, 1988; Castrucci *et al.*, 1991). More recently, experimental exposure of calves to this NCP New York-1 strain resulted in severe disease, with one calf dying and showing marked lesions, especially in lymph nodes and small intestine (Marshall *et al.*, 1998). This also included fibrinoid necrosis of arterioles, a lesion which is sometimes seen in mucosal disease but not in acute BVD (Barker *et al.*, 1993). In addition, widespread haemorrhages were seen in the viscera, a lesion usually associated with Type 2 BVDV. A complete blood count on the day before death revealed panleucopenia and thrombocytopenia (3x 10^9 platelets per L) and BVDV was isolated from nasal swabs. This report highlights the potential of a Type 1, NCP strain to cause thrombocytopenia and severe disease in the absence of persistent infection.

Studies in which calves were exposed to other NCP Type 1 BVDV isolates have resulted in either mild disease or subclinical infection (Wilhelmsen *et al.*, 1990; Bolin and Ridpath, 1992). The characterisic pathology has been described (Wilhelmson *et al.*, 1990).

Experimental challenge with Type 2 isolates has been shown to cause severe and sometimes fatal disease (Bolin and Ridpath, 1992; Ridpath *et al.*, 1994).

A study was carried out to compare the distribution of two antigenically distinct Type 2 isolates in specific pathogen-free (SPF) calves 10 days after inoculation with BVDV (Marshall *et al.*, 1996). Microscopic lesions were limited to the lymphoid tissues and

GIT. This experiment did not demonstrate a difference in distribution between the two strains, only a variation in the degree of clinical signs. Overall, the disease was mild and similar to that described in other experiments in which calves were inoculated with Type1 isolates (Marshall *et al.*, 1996). The significant feature of the experiment may be the fact that it was carried out under SPF conditions. This contrasts with the calf previously described by Marshall *et al.*(1998), which developed severe disease after inoculation with the New York-1 strain. The animal was not kept in an SPF environment, and numerous colonies of mixed bacteria, including coliforms, were found in areas of infarction around the necrotic arteries and arterioles in the submucosa of the small intestine. It is possible that these bacteria contributed to the development of the lesions, as BVDV-specific antigen was not detected in the vicinity of damaged blood vessels. The presence of other pathogens has been shown to affect the severity of the lesions resulting from BVDV infection (Potgieter, 1995; Potgieter, 1997).

Thus, the ability of NCP isolates of BVDV to cause severe clinical disease following primary acute infection in cattle remains controversial (Barker *et al.*, 1993).

The NCP strain of BVDV isolated in this study was from calf 47 in the disease outbreak (Chapter 3). This isolate was chosen for *in vivo* pathotyping to establish whether this was a strain of pestivirus capable of causing severe disease in its own right. Two further objectives were to investigate whether this strain of pestivirus could spread naturally to in-contact calves, and also to determine whether a locally available vaccine could provide protection against it.

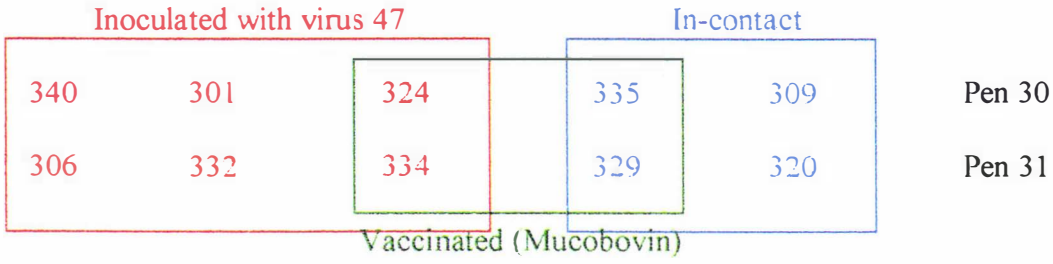
4.2 MATERIALS AND METHODS

4.2.1 Experimental design

Eleven crossbred beef calves, which tested negative for BVDV antigen and antibody, were selected from a mob of 39 weaners on a Massey University farm in the Wairarapa. Four of these calves were vaccinated against BVDV on the farm using sensitising and booster doses of "Mucobovin" (Merial, New Zealand) three weeks apart. On the 4th June, 1998, the 11 calves were transported to the experimental unit at Massey University.

Within 24 hours of arrival, calf No.141 was euthanased, necropsied and sampled as a negative control. The remaining 10 calves were housed in two separate pens. Extractor fans were installed to provide a negative pressure environment and reduce excess humidity. Eight days after arrival, six calves were randomly selected and infected with the NCP 'virus 47' strain. Four of these were unvaccinated calves (Nos. 301, 306, 332 & 340) and two vaccinated (324 & 334). Inoculation was by both the nasal and oral routes, with a total of 3.2×10^4 50% tissue culture infective doses ($TCID_{50}$) in 3.2 mL. The four non-infected animals were left in-contact; two unvaccinated calves (Nos. 309 & 320) and two vaccinated calves (Nos. 329 and 335) [Table 4.1].

Table 4.1 Experimental Design



From 12 to 21 days post-inoculation selected calves were euthanased, and at regular intervals necropsied, with appropriate samples being collected for virology, pathology and microbiology (Appendix 4.1).

4.2.2 Propagation of virus

A non-cytopathic BVDV isolate from the outbreak described in chapter 3 (virus 47) was selected for use in the transmission experiment. Virus isolation had been carried out using a buffy coat sample on Belu cell culture. Two passes were carried out, and inoculum from the second pass was again propagated in Belu cell culture, examined for CPE, harvested after one week, and stored at -70 ^{0}C.

Growth medium was removed from two flasks (80 cm^2) of Belu cells that were near confluence and 500 uL of cell culture supernatant from the second passage of isolate 47 was added to each. After adsorbing at 37 ^{0}C for 30 minutes, 20 mL of maintenance medium was added to each flask, before incubating at 37 ^{0}C and 5% CO_2 for one week. Both flasks were then frozen to -70 ^{0}C, thawed (1 hour) and the supernatant centrifuged at 600 g for 10 minutes. Two mL of the supernatant were sent to the Central Animal

Health Laboratory (CAHL) for BVDV antigen (ELISA) testing, and the rest dispensed as 5 mL aliquots and stored at -70 ^{0}C.

On the day of infecting the six calves, the virus suspension was thawed and dispensed as 24 x 800 μL aliquots in 1 mL syringes (Terumo, Tokyo). These were placed on ice, taken to the cattle, and delivered as one syringe per nostril and one syringe either side of the mouth.

4.2.3 Titration of virus

Tenfold serial dilutions of the virus were made up to 10^{-9} in a 96-well microtitre plate ("Nunclon", Denmark). These dilutions were made in 100 μL of growth media per well, with six replicates of each dilution. BVDV-free MDBK cells were trypsinised, counted and diluted to achieve a concentration of 2 x 10^5 per mL, then 100 μL added to each well. To another column of wells was added 100 μL of cells and 100 μL of medium without virus. The plate was incubated at 37 ^{0}C and 5% CO_2. Once monolayers were confluent (24-36 hours) the growth medium was replaced by maintenance medium. Fixation of this titration plate was carried out at day 4. The medium was discarded, the wells washed with 0.15 M saline, then fixed in a 20% acetone solution (see Appendix 2) for 10 minutes. The acetone was discarded and the plate dried overnight at 37 ^{0}C before sending to the CAHL for immunocytochemistry. The titre was calculated using the Karber Spearman method (Lennette and Schmidt, 1969).

4.2.3 Antigen and antibody testing

During the 6 week selection and vaccination process at the farm in the Wairarapa, blood samples were collected on three separate occasions to determine BVD antigen and antibody status using the ELISA test. This allowed selection of calves believed to be susceptible to BVDV, as well as ensuring a negative status before vaccination and prior to entry to the isolation unit at Massey University.

During the transmission experiment at Massey University, six calves were blood sampled five times, four were sampled four times, and the control calf (No. 141) was sampled once only, at the time of euthanasia (Table 4.2). Buffy coat (from heparinised blood) collected at each period was tested at the CAHL for BVD antigen using the ELISA test. Serum samples were tested at Massey University for BVD antibody using the SNT, and also at the CAHL for BVD antibody by the ELISA test. Whole blood (EDTA) was used for haematological tests at Massey University.

4.2.4 Clinical observations

The times when clinical examination was carried out have been designated as 'periods' and assigned a number (0-IV) as well as a description relating them to the time of inoculation with virus 47 (Table 4.2). In the table, the calf numbers have been listed in the order of euthanasia and necropsy.

The control calf was examined before slaughter (Period 0). The other ten calves in the experiment were observed daily, and examined clinically as a group on four separate occasions before necropsy began (Periods 0-III). Once four necropsies had been completed, the remainder were then examined individually immediately prior to euthanasia (Period IV).

Table 4.2 Clinical examination and blood sampling periods.

Period number	0	I	II	III	IV
Period description	Pre Inoc.	3 days p.i.	6 days p.i.	11 days p.i.	17-21 days p.i.
Calf Number					
141	+	-	-	-	-
340 #	+	+	+	+	-
301 #	+	+	+	+	-
335	+	+	+	+	-
324 #	+	+	+	+	-
309	+	+	+	+	+
332 #	+	+	+	+	+
306 #	+	+	+	+	+
334 #	+	+	+	+	+
329	+	+	+	+	+
320	+	+	+	+	+

Legend : p.i. = post inoculation (with virus 47)
+ = physical examination and blood collection carried out
- = no examination or blood collection (euthanased)
= calves inoculated with virus 47 (eight days after arrival)

Clinical examination was carried out with attention to cardinal signs, nasal and oral discharges and erosions, lymph node enlargement, consistency and colour of faeces, skin elasticity and hoof abnormalities.

4.2.5 Haematology

Blood samples were collected as described in Table 4.2 and submitted to the Veterinary Clinical Pathology Laboratory at Massey University for a haemogram, total and differential white blood cell concentations and fibrinogen concentrations. The

erythrocyte, leucocyte and platelet concentrations were determined automatically (Cobas Minos Vet; Roche; ABX, France). Differential leucocyte concentrations were determined by counting 100 cells on blood smears stained with Diff-Quik (Dade AG, Dudingen, Switzerland).

4.2.6 Parasitology

Faecal samples were collected from the 10 experimental calves only once, over a four day period (two calves were sampled 3 days p.i., two 4 days p.i., and the remaining six 6 days p.i.). Faecal egg counts (FEC) were carried out using a Modified McMaster egg-counting procedure. The remainder of each faecal sample was then combined with the others and a larval culture carried out to identify the strongylid eggs. After incubating at 27 ^{0}C for 7 days the larvae were recovered by the Baermann technique, identified, and the proportions determined.

At necropsy, total worm counts were carried out in five calves (Nos. 306, 320, 329, 332, 334). Abomasal contents and small intestinal contents were collected to recover the total worm burden. A representative sample (10%) was removed from the total and washed to remove obscuring debris. The worms were identified and counted in a sub-sample (10%) and the total number of worms of each genus was estimated for the total burden. As well as counting adults, 4th stage larvae were also recorded.

4.2.7 Histology

Samples collected for histology were selected and processed as described in Chapter 3. The tissues examined were scored 0 to 4 to indicate the presence of a lesion and degree of severity. [0 = no lesion; 1 = mild lesion; 2 = moderate lesion; 3 = severe lesion; 4 = severe and widespread lesion]. Although this scoring system was subjective, it was found to be repeatable on blind re-runs with a single examiner.

4.2.8 Virus isolation

Serum samples collected at 3 days and 6 days post viral inoculation were submitted to the CAHL for virus isolation, which was undertaken using the two pass test and IP staining.

Samples of spleen and mandibular lymph node were also subjected to virus isolation, using a three pass test, as described in Chapter 2.6.2.

4.2.9 Statistical analysis

A one-way analysis of variance was applied to haematologic, weight and lesion score data. Comparisons were made to determine significant differences between groups (Statistix for Windows).

4.3 RESULTS

4.3.1 Titration of virus

The titre of the virus inoculum used in the transmission experiment ('virus 47') was calculated to be 10^4 $TCID_{50}$ per mL.

4.3.2 Clinical signs

Detailed examination revealed a number of signs of acute pestiviral infection, but the changes observed were mostly minor in nature, and only of short duration.

(a) Cardinal signs.

During repeated clinical examinations, the cardinal signs were recorded (Table 4.3).

Heart rates (HR) were within the reference range in all calves at their initial examination (mean of 70 per min., range of 56 to 80). Following inoculation with virus, the mean value for all calves exceeded the reference range at both 3 and 6 days post inoculation (82 and 87 per min.). By 11 days or more after inoculation the mean values were again within the normal range. The rise in HR reached a peak at 6 days post inoculation (p.i.) in all groups. Two inoculated calves (Nos. 301 & 324) had irregular heart sounds.

Respiratory rates (RR) were just above the reference range at the initial examination (mean of 31 per min., range of 26 to 38) and then increased by approximately one third by 3 days p.i. (42 per min., range 36-52). This was similar for the sixth day p.i., and then dropped again at 11 days, but remained approximately one quarter greater than the original base value (39 per min., range 34 to 40). The inoculated calves showed the highest respiratory rates at 3 days p.i. with a mean of 44 per min. (calf 340 was measured at 52 per minute).

Table 4.3 Cardinal signs of challenged and in-contact calves.

	Heart rate (per min.)					*Respiratory rate (per min.)*					*Temperature (° C)*					*Mucous Membranes*				
Period	O	I	II	III	IV	O	I	II	III	IV	O	I	II	III	IV	O	I	II	III	IV
Group I																				
340	68	92	60	68	-	30	52	40	42	-	38.8	39.0	39.0	39.3	-	N	P	P	P	-
301	80	# 68	72	72	-	32	44	42	36	-	39.3	38.7	39.1	39.2	-	N	P	P	I	-
306	80	88	100	80	76	26	36	40	34	32	38.8	39.2	38.8	38.7	-	N	N	I	N	N
332	80	68	96	72	84	38	44	40	48	48	39.4	39.0	39.2	39.3	38.8	N	P	I	I	I
Group Mean	77	79	82	73	80	31.5	44	40.5	40	40	39.1	39.0	39.0	39.1	38.8					
Group 2																				
324	56	# 72	99	76	-	30	48	40	32	-	39.3	39.1	39.6	39.2	-	N	I	N	I	-
334	72	80	76	64	72	30	38	48	38	32	38.9	39.0	39.2	38.7	39.4	N	I	I	I	N
Group Mean	64	76	87.5	70	72	30	43	44	36	32	39.1	39.1	39.4	39.0	39.4					
Group 3																				
335	68	84	90	80	-	28	36	44	40	-	39.4	39.0	38.9	39.1	-	N	I	I	I	-
329	60	102	99	68	-	32	38	38	40	-	39.6	38.8	39.5	39.2	-	N	N	N	I	-
Group Mean	64	93	94.5	74	-	30	37	41	40	-	39.5	38.9	39.2	39.2	-					
Group 4																				
309	64	76	90	80	80	28	46	34	32	-	39.3	38.8	38.9	38.8	38.5	N	N	N	I	P
320	72	88	88	60	72	32	36	48	40	40	39.7	39.2	39.8	39.1	38.6	N	N	N	N	N
Group Mean	63	82	89	70	76	30	41	41	36	40	39.5	39.0	39.4	39.0	38.6					
Average(all)	**70**	**82**	**87**	**72**	**77**	**31**	**42**	**41.5**	**39**	**38**	**39.3**	**39.0**	**39.2**	**39.1**	**39.0**					
Range (all)	56-80	68-102	60-100	60-80	72-84	26-38	36-52	34-48	34-40	32-40	38.8-39.7	38.7-39.2	38.8-39.8	38.7-39.3	38.5-39.6					
Reference	40-80	40-80	40-80	40-80	40-80	10-30	10-30	10-30	10-30	10-30	38.5(39.4)	38.5(39.4)	38.5(39.4)	38.5(39.4)	38.5(39.4)					

LEGEND

Group 1 = Unvaccinated, challenged
Group 2 = Vaccinated, challenged
Group 3 = Vaccinated, in-contact
Group 4 = Unvaccinated, in-contact

Period 0 = Pre inoculation
Period I = 3 days post inoculation
Period II = 6 days post inoculation
Period III = 11 days post inoculation
Period IV = 17- 21 days p.i.

# = irregular heart rate
N = normal mucous membranes
P = pallor of mucous membranes
I = injected mucous membranes

The mean temperature for all calves was relatively high at the initial examination (39.3 ^{0}C, range 38.8-39.7 ^{0}C) and remained between 39.0-39.2 ^{0}C throughout the experiment. Of 26 recordings in the inoculated animals, only 3 (11.5%) reached or exceeded 39.4 ^{0}C. Of 18 recordings in the in-contact calves, 5 (28%) exceeded this 'critical temperature'.

Before inoculation with virus the mucous membranes (oral, conjunctival and vulval) were examined and found to be normal. Three calves in Group 1 (Nos. 301, 332 and 340) subsequently showed pallor of the mucous membranes on examination three days after inoculation. This remained constant in subsequent examinations in calf 340, while changing to injected (reddened) membranes by 6 days p.i. in calf 332 and at 11 days in calf 301. The vaccinated calves (Nos. 324, 329, 334, 335) showed varying degrees of injected mucous membranes (Table 4.2). One in-contact calf (No.309) had normal, pink, mucous membranes up to 6 days p.i., but by 11 days they became injected, and by 16 days p.i. pallor had developed.

(b) Growth rate.

Each of the calves was weighed twice on the farm (at a 21 day interval), on the day before inoculation at Massey University (14 days later) and on the day of necropsy (a varying time interval). The mean weight gain for the whole group was calculated for each of these three periods (Appendix 4.2). While on pasture the average growth rate was 800 gm per day. This dropped to 314 gm per day in the subsequent two weeks. This period consisted of a week on the farm when the calves were changed to a hay and pellet diet, followed by a week settling down in the isolation facility at the university. During the period following inoculation, there was a further drop in the mean growth rate, to 186 gm per day.

To investigate the effect of viral challenge, body weights prior to inoculation were compared with weights at necropsy, and the weight change converted to a weight gain (in gm/day) by taking into account the variable time for each calf to necropsy (Table 4.4). Since the final weights of the two unvaccinated in-contact calves were not available, comparison of the four unvaccinated, challenged calves was made with the four vaccinates. A mean for these two groups was calculated, and it was found that following inoculation with 'virus 47', the growth rate of the vaccinates was 674 gm/day, compared with 86 gm/day for the unvaccinated, challenged calves. This was not found to be statistically significant ($p = 0.1165$). Two of the vaccinated calves (Nos. 324 and 334) were also challenged, but still achieved very good growth rates (1000 gm/day and

600 gm/day, respectively). These two were compared with the four unvaccinated, challenged calves, but the difference was again not statistically significant (p =0.1978). One of the unvaccinated, challenged calves (No. 340) lost weight over the 13 day period after inoculation (-10 kg, or -770 gm per day). In the two weeks before challenge she had gained +12 kg (or +860 gm per day). The weight loss in this calf contributed markedly to the low mean weight gain of the unvaccinated challenged calves.

Table 4.4 Mean weight gain of calves following inoculation.

Calf Number	Body Wt. (Kg) At Inoculation	Body Wt. (Kg) At Necropsy	Wt.change (Kg)	Days p.i. (Days)	Wt. gain p.i. (Grams per day)
340	196.0	186.0	-10.0	13	- 769
301	223.0	225.0	+ 2.0	14	+143
306	202.0	213.0	+11.0	20	+550
332	187.0	195.0	+ 8.0	19	+421
Mean					+86 gm/day
324	189.0	204.0	+15.0	15	+1000
334	196.0	208.0	+12.0	20	+600
335	200.0	210.0	+10.0	15	+667
329	165.0	174.0	+ 9.0	21	+429
Vacc.mean					+674 gm/day
309	212.0	-		18	-
320	216.0	-		22	-
141	-	-		-	-

(c) Diarrhoea

Five calves, two unvaccinated, challenged (Nos. 301 and 340), one vaccinated, challenged (No.324), one vaccinated, in-contact (No. 335) and one unvaccinated in-contact (No.309), developed soft or loose faeces at varying times during the experiment. The faeces of the rest of the calves remained firm. No signs of clinical dehydration were detected in any calf throughout the experiment.

(d) Ocular and nasal inflammation and discharges

One calf (No. 309) had a nasal discharge at the initial examination and which continued throughout the experiment (Table 4.5). Ocular or nasal inflammation or discharges were not present in the other calves at the initial examination, but they developed following challenge. Within 3 days, two animals (Nos. 324 and 335) were found with inflammation of the nasal mucosa, one having a nasal discharge as well (No. 324). By 6

days p.i., nine animals displayed nasal or ocular abnormalities - seven had nasal discharges (three serous and four mucoid), while two others had conjunctivitis. Five of these animals continued to show clinical signs at 11 days p.i. (two serous nasal discharge, two rhinitis, and one ocular discharge) while another new case with a nasal discharge was identified (Table 4.5). On the last examination, one calf (No. 332) had developed a serous ocular discharge along with a continuing nasal discharge.

Ocular and nasal signs had largely resolved by the time of necropsy in six calves.

(e) Oral erosions and ulcers.

At initial examination, erosive lesions were not detected in any calf, except calf No. 335 which had minor superficial ulceration of the gums behind the incisor teeth. Five of six inoculated calves developed erosions in the mouth within 11 days p.i. (Table 4.5). Only one (No. 329) of the four calves that were not challenged developed erosive lesions at any time p.i.. (Table 4.5). Erosions were found in the hard palate (four cases), gum (two cases), tongue (two cases) and lip (one case). In addition, two of the inoculated calves (Nos. 332 and 340) showed marked erythema of the hard palate. All the lesions seen were minor ulcers or erosions and most were resolving by the time of necropsy.

(f) Lymph node enlargement.

Palpation of mandibular and prescapular lymph glands was routinely carried out at each examination. All 6 inoculated calves developed enlargement of lymph nodes, as did two of four calves which had not been challenged (Table 4.5). Lymph node enlargement was detected in 5 calves at 6 days p.i., with two more at 11 days p.i., and one at 18 days p.i. (Table 4.5).

(g) Hoof lesions.

No lameness nor any hoof lesions (including the interdigital cleft and coronary band) were detected in any calf during the experiment.

(h) Time course of clinical signs following viral challenge.

There was a general increase in heart rate and respiratory rate by 3 days p.i., while ocular and nasal discharges were widespread (9 of 10 calves) by 6 days p.i.. Erosions and/or ulcers were seen in 5 of 6 challenged calves at 11 days, while lymph node enlargement was evident in 8 of 10 calves at 18 days. Mild diarrhoea was seen at varying times in 5 calves, but was not restricted to the inoculated calves.

Table 4.5 Other clinical signs in experimental calves

	Ocular & nasal signs					*Oral ulcers & erosions*					*L. N. enlargement*				
Period	O	I	II	III	IV	O	I	II	III	IV	O	I	II	III	IV
340	-	-	+	-		-	+	-	-		-	-	+	+	
301	-	-	+	-		-	+	-	-		-	-	-	+	
306	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+
332	-	-	+	+	+	-	+	-	-	-	-	-	-	-	+
324	-	+	+	+		-	-	+	-		-	-	+	+	
334	-	-	+	+	-	-	+	+	+	-	-	-	+	+	+
335	-	+	+	+		+	-	-	-		-	-	+	+	
329	-	-	-	+	-	-	-	+	+	-	-	-	-	-	
309	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+
320	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
141	-					-					-				

Legend

[+] = clinical signs observed; [-] = clinical signs not observed

[ ] = no clinical observation - calf since euthanased

4.3.3 Clinical pathology

The haemogram (Complete Blood Count) for each calf over each period sampled is in Appendices 4.3 to 4.5.

(a) Red blood cells (RBC). [Appendices 4.3.1 and 4.3.2]

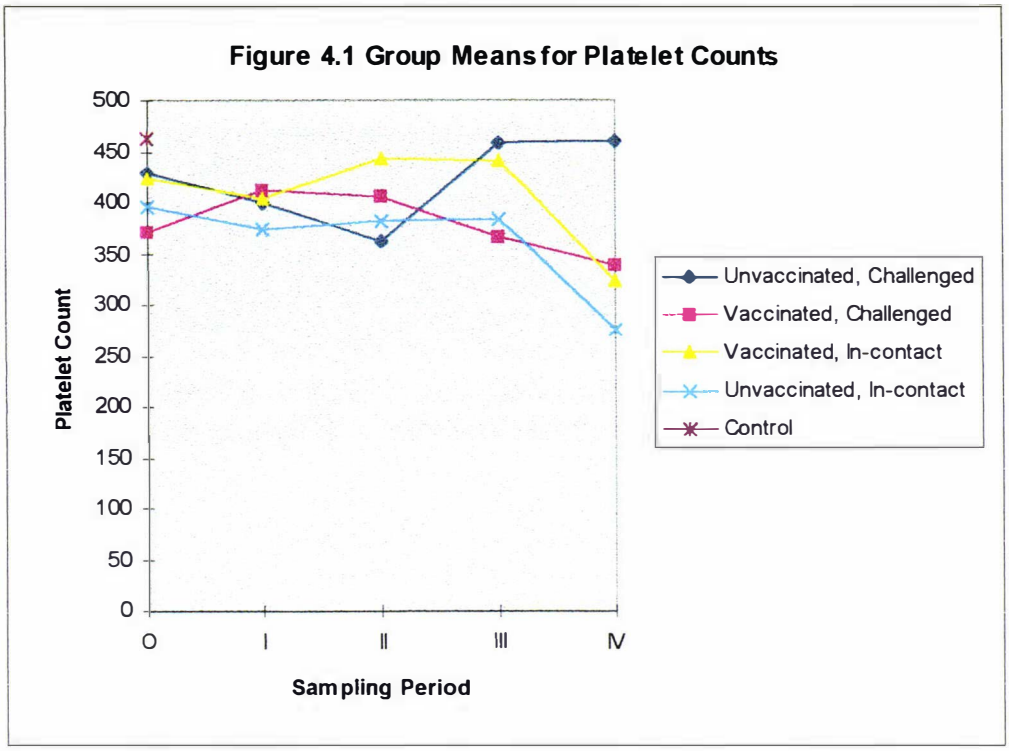
All RBC concentrations in all calves were within the reference range, and no obvious differences between groups were seen, nor any trends in successive samplings of individual calves. Other indices were also unremarkable.

(b) Platelets [Appendices 4.4.1 and 4.4.2]

Of 47 platelet tests undertaken, only one was outside the reference range (the last test of calf 309). However, observation of tests within each treatment group, revealed a distinct pattern. When the mean value for each group at each sampling period was determined (Table 4.6), the unvaccinated, challenged group showed a drop in platelet count at 3 and 6 days p.i. (periods I & II) before recovering at 11 days p.i. and beyond (periods III & IV). In contrast, the vaccinated and challenged group showed an intial rise in platelet concentration 3 and 6 days p.i. before reducing to pre inoculation values. The two groups in-contact (not challenged) showed only minor variations up to 11 days PV, whereas samples taken at the time of necropsy (period IV) were greatly reduced in platelet numbers. These changes have been represented graphically (Figure 4.1).

Table 4.6 Group Means for Platelets.

	PLATELET COUNTS x 10^9 per L				
SAMPLING PERIOD	O	I	II	III	IV
Unvaccinated, Challenged	430	400	363	458	460
Vaccinated, Challenged	370	411	406	366	339
Vaccinated, In-contact	423	403	444	442	324
Unvaccinated, In-contact	397	375	383	384	276
Control	462				



(c) Fibrinogen

All tests were within the reference range.

(d) Total Solids

Mean concentrations for the inoculated calves were marginally low, with three of six calves being consistantly low or marginal over successive tests. Two of the four in-contact calves also showed multiple marginal or low tests.

(e) White Blood Cells (WBC) [Appendices 4.5.1 and 4.5.2]

Only 25 tests out of 564 (4.4%) were outside the reference ranges for each analyte.

Total WBC

Of 47 total WBC counts, only 2 were outside the reference range. [Calf 306 had an initial marginally high count before challenge, while calf 309 (unvaccinated, in-contact), showed one high count associated with a neutrophilia]. However, consistent changes were seen in the total WBC counts over successive tests in individuals, so the mean values were determined for each group (Table 4.7).

The total WBC counts fell sharply at 3 days p.i. in the "unvaccinated, challenged" group, while the other three groups slightly increased. At 6 days p.i. these other groups had fallen to a point below their pre-inoculation value. All groups began to rise by 11

days p.i., and at the last sampling were at a level similar to their initial test. These changes are illustrated in Figure 4.2.

Table 4.7 Group Means for White Blood Cells.

	Total W.B.C.s x 10^9/L				
PERIOD	O	I	II	III	IV
Unvaccinated, Challenged	11.8	7.5	7.1	8.9	10.8
Vaccinated, Challenged	8.7	10.3	6.4	7.9	8.8
Vaccinated, In-contact	10.2	11.0	9.9	10.1	11.6
Unvaccinated, In-contact	10.4	12.5	9.7	9.9	10.0

Differential WBC Counts

Examination of the differential WBC counts revealed a consistent pattern of change in the lymphocyte component for each group (Table 4.8 and Figure 4.3).

Table 4.8 Group Means for Lymphocyte Counts.

	LYMPHOCYTES x10^9/L				
PERIOD	**O**	**I**	**II**	**III**	**IV**
Unvaccinated, Challenged	7.4	4.4	4.3	6.0	6.4
Vaccinated, Challenged	5.9	6.0	3.8	5.6	6.0
Vaccinated, In-contact	6.5	7.1	5.7	5.9	6.7
Unvaccinated, In-contact	6.9	7.5	6.8	6.3	6.1

Changes in the numbers of other WBCs were minor, with only three tests (6.4%) showing a neutrophilia and two tests (4.3%) a monocytosis. All tests for eosinophils were within the reference range. Twelve tests (25.5%) for basophils were marginally high in 8 of 11 calves. Two of the inoculated calves plus the control remained within the reference range.

Figure 4.2 Group Means for W.B.C. Counts

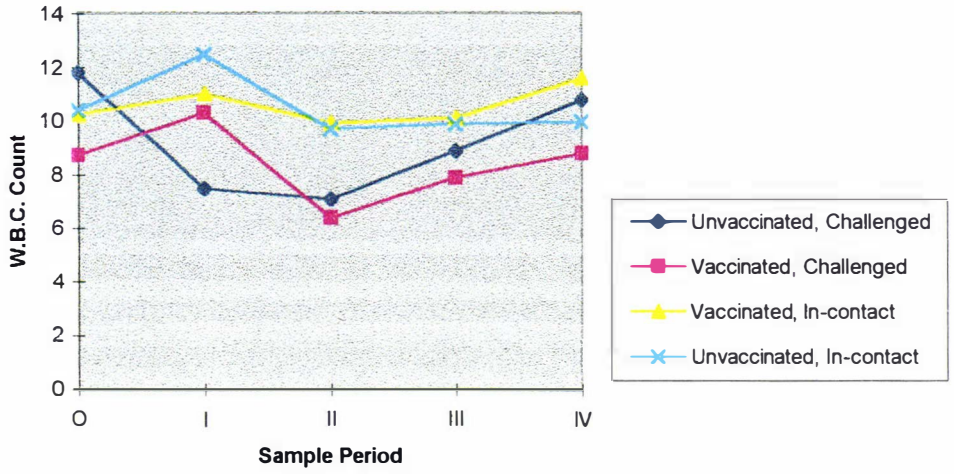
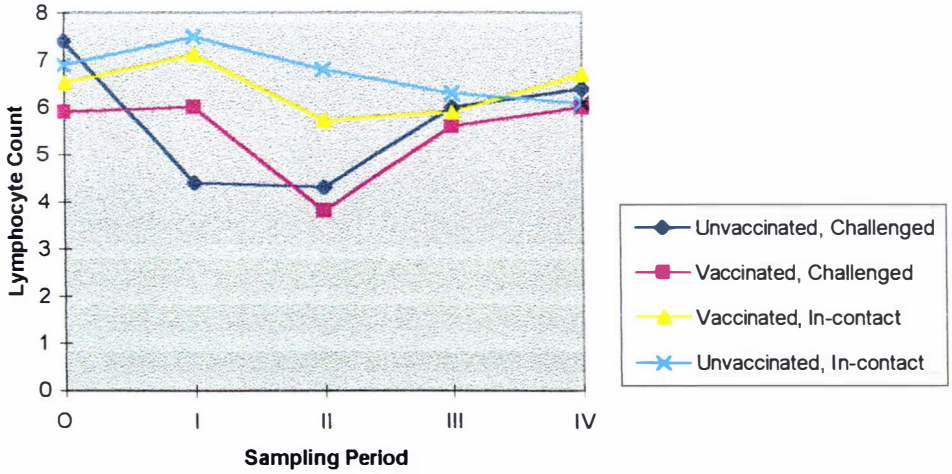


Figure 4.3 Group Means for Lymphocyte Counts



4.3.4 Necropsy

The gross lesions seen at necropsy are summarised in Table 4.9.

(a) The upper alimentary tract - mouth to omasum.

Erosive lesions were observed at necropsy in 7 of the 11 calves and these were counted (Table 4.9) and represented graphically (figure 4.4a).

Of the six calves challenged, four had erosions on the tongue and five had erosions in the oesophagus (Figure 4.5).

Of the four unvaccinated, challenged calves, two (Nos. 340 and 301) had multiple (25) small (0.5-1 mm diam.) oesophageal erosions, while the other two had fewer (6) erosions. One of these (No. 332) had small discrete erosions, while the other (No. 306) had three linear erosions, approximately 20 mm long, as well three punctate erosions. Two of these four calves (Nos. 301 and 332) also had single discrete (2-3 mm diam.) erosions in the tongue, while calf No. 306 had three small (0.5 mm diam.) tongue erosions.

Of the two calves that were vaccinated and challenged, one had no oesophageal erosions (No. 334), while the other (No. 324) had numerous (20), multifocal, small (0.5 mm), but very superficial erosions. A healed erosion was also present on the tongue of the second calf.

Of the four in-contact calves, only one had oesophageal and tongue erosions.

In the two vaccinated, in-contact calves, no erosions were observed in one (No. 335) while the other (No. 329) had healed erosions (2) on the ridges of the hard palate, healed tongue erosions (2) and one linear and one discrete erosion in the oesophagus.

In the two unvaccinated, in-contact calves, no oesophageal or buccal erosions were observed, but calf No.320 had one ulcer on a rumen pillar measuring 2.5 mm in diameter.

In the control calf (No. 141), no erosions were observed.

(b) The abomasum.

In the abomasum, lesions were found in four calves, only one of which was challenged. Multifocal small (0.5-1 mm diam.) nodules were found in the fundic abomasum of calves 309, 324 and 335, while in calf 320 multiple moderately sized ulcers (3-4 mm diam.), with an irregular shape, were found in both the fundic and pyloric regions.

Table 4.9 Gross lesions seen at necropsy of experimental calves.

Calf Number	Nos. of erosions in UAT	Red spots in nasal mucosa	Purulent discharge in RT	Collapse / consolidation in lung	Enlarged lymph nodes	Abnormal intestinal contents	Congestion of intestinal mucosa	Parasitic nodules	Other findings
340	25	+++	Trachea		Mandibular	Mucoid (doud.) Watery (jej.)			
301	26	+++		Anteroventral Lobes	Mandibular	Watery (jej.)			G.B. engorged Liver friable
332	7	+++	Nasal cavity Bronchi				Distal jejunum		Palatine Tonsils enlarged & haem.
306	9	++			Prescapular Mandibular (#)	Mucoid (jej.)	Proximal ileum		G.B. engorged
324	21	+				Muciod (duod.) Watery (jej.)	Duodenum & jejunum	Abomasum (1 mm)	
334	0	+			Mandibular				
335	0	++			Mandibular			Abom. (1 mm) Ileum (3 mm)	G. B. engorged Liver 'marbled'
329	6	++			Retropharyngeal (abscessed)		Distal jejunum		S/c petechial haemorrhages
309	0	o		Anteroventral Lobes				Abom. (1 mm) Il/colon (3mm)	Enlarged thymus G.B. engorged
320	1	o		Anteroventral Lobes					Ulcers in abomasum
141	0	o	Trachea Rt. Bronchus	Post. Lobe Rt. Lung					

Legend :- UAT = upper alimentary tract; RT = respiratory tract; G.B. = gall bladder; S/c = subcutaneous.
o = nil; + = few; ++ = many; +++ = multiple
(#) = haemorrhagic

(c) The respiratory tract.

Nasal haemorrhages.

Pin-point, circular haemorrhages were found in the nasal mucosa of all six challenged calves and in two of the in-contact calves (Figure 4.7).

These petechiae were densely multifocal in the four unvaccinated, challenged calves (eg 5-6 per sq. cm in calf 332), while the two vaccinated, challenged calves showed only a few (3-4 in total).

The two vaccinated, in-contact calves had numerous pin-point focal haemorrhages in the nasal mucosa, while the two unvaccinated, in-contact calves were unaffected, as was the control calf.

Purulent discharge.

Purulent discharge was present in the respiratory tract of three calves. Two were challenged (Nos. 340 and 342) and the third was a control (No. 141), which had a considerable amount of tenacious yellow pus in the lower trachea extending into the right bronchus.

Lung lesions.

The control calf (No. 141) had a small (10 mm diam.) firm area of consolidation in the posterior lobe of the right lung.

(d) The lymph nodes.

The mandibular lymph nodes were enlarged in five calves; four challenged (Nos. 340, 301, 306, 334) and one non-challenged (No. 335). The mean dimensions for seven glands measured was: 45 mm long, 29 mm wide, and 14 mm thick. Prescapular lymph nodes were also enlarged (70 x 25 mm) in one calf (No. 306). Lymph nodes of this size are expected in normal adult cattle (Sisson and Grossman, 1953).

Occasional petechial haemorrhages were seen in the cortex of one enlarged mandibular lymph node from calf 306.

An enlarged retropharyngeal lymph node from calf 329 contained a 3 mm diam. abscess in the medulla.

Figure 4.4a Lesions which appeared to be erosions on gross examination.

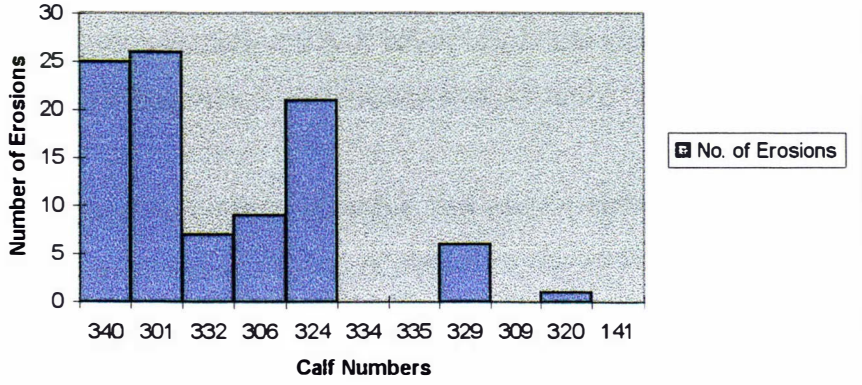
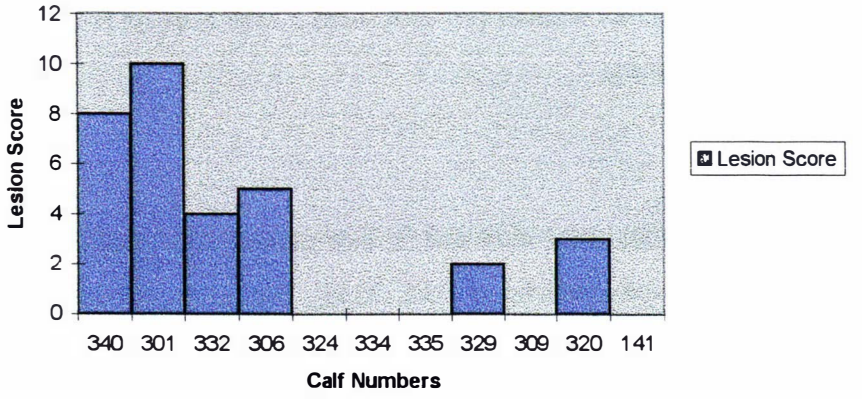


Figure 4.4b Erosive lesions confirmed on microscopic examination.



(e) The intestine.

The contents of the small intestine were excessive in four of the six challenged calves. In two calves (Nos. 340 and 324) the duodenal contents were mucoid, while the jejunal contents were watery. The jejunal contents were also watery in calf 301, and mucoid in calf 306.

Congestion of the intestinal mucosa was only seen in four of the eleven calves. This involved the duodenum and jejunum in one calf (No.324), and the distal jejunum or proximal ileum in the other three (Nos. 324, 329, 332).

Of three calves (Nos. 324, 335, 309) which had small (0.5-1 mm) abomasal nodules typical of ostertagiasis, two also had larger (3 mm) nodules in the ileum (Nos. 309 and 335) while one had similar nodules in the colon (No. 309). Two to three nodules were seen per 10 cm of intestine and they were firm and palpable from the serosal surface of the ileum. They were likely to be due to invasion by *Cooperia* species.

(f) Other findings.

The gall bladder was distended in four calves, providing evidence for inappetance in these animals. Two of these also had a 'marbled' liver capsule, while one liver was friable.

The palatine tonsils were enlarged (50 mm long) and haemorrhagic in one challenged calf, number 332 (Figure 4.6).

The thymus was very large (200 x 80 cm) in a non- challenged non-vaccinated calf (No. 309).

Subcutaneous petechial haemorrhages were seen throughout the carcase of calf 329 (this calf showed a dramatic fall in platelet count from 441 at 11 days p.i., to 202 at the time of necropsy).

4.3.5 Histopathology

(a) The upper alimentary tract (mouth to omasum).

The lesions observed were scored according to their severity and summed for each calf, and this data is summarised in Table 4.10. Some of the erosive lesions observed at necropsy were not confirmed histologically, and this is shown graphically in Figure 4.4.

Erosions and epithelial necrosis (often found together) were the lesions most commonly seen, both in terms of the number of animals affected and also when expressed as the

Table 4.10 Scores for histological lesions in the squamous epithelium of the upper alimentary tract.

Calf Number	Epithelial inflammation	Epithelial necrosis	Cleavage vesicles	Erosions	Ulceration	Submucosal inflammation	Parakeratosis	Hyperkeratosis	TOTAL SCORE
340	1	1	0	4	4	0	1	1	12
301	0	7	0	7	3	5	1	1	24
306	3	0	0	5	0	3	2	2	15
332	4	7	2	0	4	2	0	0	19
324	0	0	0	0	0	0	2	2	4
334	0	0	0	0	0	0	0	0	0
335	0	0	0	0	0	0	0	0	0
329	0	1	2	2	0	0	0	0	5
309	0	1	0	0	0	0	0	0	1
320	0	0	0	0	3	1	0	0	4
141	0	0	0	0	0	0	0	0	0
TOTAL	8	17	4	18	14	11	6	6	84

total scores of all the calves. The oesophagus was the most common site affected, followed by the tongue and the hard palate (Figure 4.9).

The four calves challenged and not vaccinated (Nos. 340, 301, 306 and 332) showed the most lesions and had the highest scores (12, 24, 15 and 19 respectively). The total score for this group (70) represented 83% of the grand total for all the eleven animals. Most erosions seen in these animals involved necrosis of the stratum corneum (Figure 4.9), while some extended to the stratum granulosum or stratum spinosum. Fungal hyphae and pockets of bacteria were commonly present in these necrotic areas, while inflammation was usually seen in the underlying dermis (Fig. 4.9), with neutrophils the main cell present, along with some macrophages and plasma cells. Erosive lesions were seen in the oesophagus of calf 306 adjacent to areas of hyperkeratosis and parakeratosis. Where ulcers were present, they extended through the basement membrane (stratum basale) with neutrophils predominating in the central area of necrosis, while the adjacent epidermal cells often showed hydropic degeneration. Cleavage vesicles were seen in the papillae in the reticulum and rumen of one calf (No. 332).

Of the four vaccinated calves, two had no observable lesions (Nos. 334 and 335), while the other two (Nos. 324 and 329) had low scores of 4 and 5 respectively. In calf 324, where 20 small (0.5 mm) superficial spots had been observed throughout the oesophagus at necropsy, eight sections of oesophagus were examined histologically for lesions. Rough, thickened areas in the stratum corneum (hyperkeratosis) where keratinocyte nuclei remained (parakeratosis) were observed, but no erosions were found (Figure 4.4b). In calf 329, an area of superficial focal necrosis was observed in the epidermis in the oesophagus, beneath which was a zone of parakeratosis. Squames were present above this, with some bacterial invasion. In a rumen pillar of this calf, two discrete microabscesses were present in the stratum spinosum of the epidermis, but no erosive lesions were present in the hard palate or tongue.

The two unvaccinated, in-contact calves (Nos. 309 and 320) had low scores (1 and 4 respectively). Calf 309 had a single small focus of epithelial necrosis in one rumen section, but no erosions were evident in sections of dental pad, tongue, oesophagus, reticulum or omasum. Calf 320 had a large ulcer on a rumen pillar. Fibroblast proliferation and vascularistion was evident at the base of the ulcer, indicating an advanced, organising lesion.

The control calf (No. 141) showed no lesions in the squamous epithelium of the upper alimentary tract.

Table 4.11 Scores for histological lesions in the abomasum.

Calf Number	Mucosal congestion	Mucosal infiltration	Epithelial metaplasia	Gland necrosis	Follicular hyperplasia	Globular leucocytes	TOTAL SCORE
340	2	2	3	3	3	2	15
301	0	0	0	0	0	0	0
306	0	2	0	0	0	0	2
332	2	3	0	0	0	0	5
324	0	0	0	0	4	0	4
334	0	2	0	0	3	0	5
335	0	3	0	0	0	0	3
329	0	0	0	0	0	0	0
309	0	1	0	0	0	0	1
320	2	3	0	0	0	2	7
141	0	2	0	0	3	0	5
TOTAL	6	18	3	3	13	4	47

(b) The abomasum

The number of calves affected and the score of each lesion is recorded in Table 4.11.

The main lesion seen (8 of 11 calves), was mucosal infiltration by inflammatory cells, with a range of severity from mild to severe. The inflammatory cells were mainly in the lamina propria, with eosinophils and plasma cells predominating, along with some lymphocytes. In addition, globular leucocytes were present within the epithelium of two calves and also within submucosal lymphoid follicles in one of these.

Hyperplasia of mucosal lymphoid follicles also scored highly, with 3 calves (Nos. 141, 334, and 340) showing severe lesions while another (No. 324) had extremely hyperplastic follicles with very active mitotic figures (four / three high power fields).

In sections of abomasum from the control calf (No. 141), a nematode (probably *Ostertagia spp)* was seen in one gland, along with eosinophils and multinucleate giant cells. This, together with the presence of hyperplastic lymphocytic nodules, indicated significant parasitic involvement. Four nematode larvae (probably *Ostertagia spp.)* were seen in the abomasal lumen of calf 306.

The total score for one of the challenged calves (No. 340) was high, with a score for every abomasal lesion recorded.

(c) The intestine.

The main histological findings are summarised in Table 4.12.

Infiltration of the intestinal mucosa (lamina propria) by inflammatory cells was a lesion common to all calves, including the unexposed control animal, and resulted in the highest total histological score. This infiltration ranged from moderate to severe, and the proportion of sections where eosinophils predominated was 72.5%, plasma cells 15.0%, lymphocytes 7.5% and globular leucocytes 5.0% (see Appendix 4.6 for details). The widespread presence of eosinophils (in all but three sections) was likely to be due to the presence of nematodes, as these were seen in sections from seven of the eleven calves. In two calves (No. 301 and 306) worm larvae (6 in total) were seen in the intestinal crypts. In four calves (Nos. 332, 324, 334 and 335) *Cooperia* nematodes were seen in the lumen of the small intestine. In addition, the nodules seen in the ileum of calves 309 and 335 at necropsy were confirmed histologically to be large parasitic granulomas

Table 4.12 Scores for histological lesions in the intestine.

Calf Number	Mucosal congestion	Mucosal infiltration	Epithelial hyperplasia	Follicular hyperplasia	Cryptal necrosis	Lysis of Peyer's patches	Inactive Peyer's patches	Cryptal prolapse	Denuded villi	Globular leucocytes	Submucosal inflammation	TOTAL SCORE
340	0	2	4	0	3	2	4	0	0	0	0	15
301	0	3	0	0	0	2	4	3	3	0	0	15
306	0	2	0	0	0	3	4	3	0	0	0	12
332	3	3	0	0	1	1	4	0	0	1	0	13
324	3	3	1	0	0	4	4	1	0	0	2	18
334	0	2	0	1	2	0	0	0	0	0	0	5
335	1	3	0	0	0	0	0	0	0	0	0	4
329	3	2	1	0	0	2	1	0	0	3	1	13
309	0	2	0	4	0	0	0	0	0	0	0	6
320	0	2	0	1	0	0	0	0	0	0	0	3
141	0	2	0	0	0	0	0	0	0	0	0	2
TOTAL	10	26	6	6	6	14	21	7	3	4	3	106

present in the submucosa. One large lymphocytic nodule was seen where a worm had penetrated the muscularis mucosa, while in another nodule, four worm larvae were observed in a lymph vessel from which there was leakage of fibrin. Surrounding the larvae were large numbers of macrophages, and eosinophils.

In one calf (No. 306) coccidia were seen in the intestinal crypts, while in another (No. 320) a protozoal oocyst and a gametocyte were seen in the villus epithelium of the small intestine.

The next most common lesions were seen in the Peyer's patches (Pp). Five of the six challenged calves (Nos. 340, 301, 306, 332 and 324) showed lymphocyte depletion (lysis of Pp) along with reduced activity, as measured by the number of mitotic figures (Figure 4.10). The degree of lymphocyte depletion ranged from mild to severe, and no mitotic figures could be found in Pp of these five animals. This contrasted with the control calf (No.141) where Pp were active (8 mitotic figures seen per high power field) and packed with lymphocytes (Figure 4.11). One of these four inoculated calves was also a vaccinate (No. 324), yet there was extreme depletion of Pp, leaving an eosinophilic amorphous material remaining. There was no mitotic activity evident, and mild prolapse of crypts into Pp was observed (a lesion typical of BVDV infection).

One in-contact calf (No. 329) that was vaccinated showed moderate lysis, yet reasonable mitotic activity (5-6 figures per high power field). The Pp's appeared enlarged, the presence of macrophages gave a "starry sky" appearance, and there was a small area of necrosis in one germinal centre. These lesions suggested that an active response to BVDV infection was occurring in this animal.

The distinctive lesions of BVDV infection, namely cryptal necrosis and cryptal prolapse (Figure 4.12), were seen in all the six challenged calves (one lesion or the other), but were absent from the unchallenged calves.

Mucosal congestion was only seen in four of eleven calves, two challenged calves (Nos. 332 and 324) and two in-contact vaccinates (Nos. 335 and 329).

Other intestinal lesions were seen at low frequency, and did not appear to be related to the viral challenge. These included epithelial hyperplasia, follicular hyperplasia, denuded villi, and submucosal inflammation.

When the histological score of each lesion was totalled for each calf, five of the six challenged calves showed scores in double figures, which sum to more than two-thirds of the grand total (73 of 106) for all calves. The only other calf to reach double figures

Table 4.13. Scores of histological lesions in lymph nodes.

Calf Number	Paracortical hyperplasia	Lymphocyte depletion	Inactivity	Abscessation	Haemorrhage	TOTAL SCORE
340	4	2	4	0	0	10
301	2	2	3	2	1	10
306	7	8	3	1	1	20
332	13	9	5	0	0	27
324	1	0	0	0	0	1
334	2	1	3	0	0	6
335	3	0	6	0	0	9
329	2	0	2	4	0	8
309	0	0	0	0	0	0
320	0	0	1	0	0	1
141	0	0	0	0	0	0
Totals	34	22	27	7	2	92

Note :- Scores have been summed where multiple lymph nodes were examined.

was No. 329, and lesions in Pp indicate that there may have been spread of BVDV infection to this in-contact animal.

(d) The lymph nodes.

All the lesions seen on histological examination were scored, and the results are summarised in Table 4.13.

The results for all the lymph nodes examined were summed and recorded for each calf. This included mainly mandibular and mesenteric nodes, which were collected routinely, and also prescapular, hepatic, ileal, retropharyngeal and mediastinal nodes which were collected when lesions were observed grossly. The lesions recorded were found to be shared by most of the glands, possibly indicating that the effect on lymph nodes was a systemic one.

Increased numbers of lymphocytes in the paracortex was the lesion with the highest score (Figure 4.14). This paracortical hyperplasia was a feature of both the challenged and vaccinated calves, but was not seen in the unvaccinated, in-contact calves, nor in the control (Figures 4.13 and 4.14).

Depletion of lymphocytes in the germinal centres was seen in five of the six challenged calves, but was not seen in the unchallenged ones. Of the two vaccinated and challenged calves, No. 324 was not depleted of lymphocytes, and No. 334 showed only mild depletion.

Cellular activity in the germinal centres was gauged by counting mitotic figures. Only cells obviously undergoing mitosis were counted per three high power fields. Eight mitotic figures were found in three high power fields in lymph node sections from the control calf (No. 141), and this was found to be repeatable over different germinal centres and lymph nodes. In terms of "inactivity", this was allocated a score of zero. At the other end of the scale, one challenged calf (No. 340) was found to have no mitotic figures in germinal centres. This animal was considered to have severe and widespread inactivity of germinal centres and was scored as 4. The results obtained using this scoring system were as follows :-

Count of Mitosis	Score of Inactivity		Calf Number
0	4	Complete inactivity	340
1-2	3	Severe inactivity	329
3-4	2	Moderate inactivity	301, 306, 332, 334, 335
5-6	1	Mild inactivity	320
7-8	0	Fully active	324, 309, 141

The results (Table 4.13) show that reduced activity of germinal centres is a significant lesion in this experiment, scoring second to paracortical hyperplasia when scores for all lymph nodes are summed. Seven calves showed moderate to complete inactivity in germinal centres. This included 5 of the 6 challenged calves, and the 2 vaccinated, in-contact calves. Of the unvaccinated, in-contact calves, No.320 displayed mild inactivity of germinal centres while No. 309 showed full activity. One of the vaccinated and challenged calves (No. 324) showed activity levels equivalent to the control calf (No.141).

When the total scores for each calf are compared, the four unvaccinated, challenged calves are seen to have the highest scores, indicating the greatest number and extent of lymph node lesions. The summed score of this group is 73% of the grand total, while sum of the four vaccinates is 26% and the two in-contacts plus the control make up the remaining 1%.

(e) The spleen

In calf 332, there was reduced cellular activity in the white pulp areas (periarteriolar sheaths) which was moderate in degree (scored at 2). In addition, this unvaccinated, challenged calf had the highest score of any calf for lymph node lesions, as well as being the only calf with lesions in the thymus [see (g)].

(f) The tonsil

Of the six calves which had lesions in the palatine tonsils, five were challenged calves. Three of these (Nos. 340, 332 and 301) showed tonsillar hyperplasia, with excessive necrotic debris in crypts (Figure 4.16). In addition, germinal centres in calf 340 showed reduced numbers of mitoses and in calf 332 contained some haemorrhages, while in calf 301 some haemorrhage was present in the lympho-epithelium. In calf 306 there were some reactive areas in the tonsil with keratin pearls in the periphery.

One of the challenged and vaccinated calves (No. 324) presented a different picture, with wide lympho-epithelial layers bounding the crypts. These contained central areas of necrotic neutrophils, surrounded by a zone of macrophages, then epithelial cells which were producing numerous keratin pearls. These lesions were considered indicative of increased tonsillar function and were interpreted as a subacute tonsillitis.
Some haemorrhage was seen in the lympho-epithelium lining the crypts in calf 335, a vaccinate in-contact, while no lesions were seen in the remaining animals.

(g) The thymus

One calf (No. 309) had a large thymus and in another (No. 335) the T-lymphocytes appeared quite dense. However, these were not considered to be lesions, but rather variations of normal thymic activity. In calf 332 (unvaccinated, inoculated) the thymus was congested, haemorrhages were seen in the areas of Hassell's corpuscles, while some germinal centres showed necrosis and depletion.

(h) The nervous system.

The areas of the nervous system routinely examined in each calf were cerebrum (anterior, middle and posterior sections), hippocampus / thalamus, cerebellum, pons, medulla oblongata (obex) and spinal cord (cervical, thoracic and lumbar).
The lesions seen were mild in nature, except for the moderate cuffing seen in the hippocampus in one unvaccinated, challenged calf (No. 340). In the other calves, mild perivascular cuffing by mononuclear cells was the most common lesion seen, with the cerebrum the most common site. Eleven points of the total score of fifteen (73%) were found in the challenged calves.
Other lesions seen were some small haemorrhages in the pons of calf 334, and an increased cellularity in the cerebellar meninges of calf 324, both vaccinated, challenged animals.
No lesions were recorded in the spinal cord sections.
No lesions were seen the nervous tissues of four calves, two challenged (Nos. 301 and 332) and two not challenged (Nos. 329 and 320).
Appart from the haemorrhages seen in the hypothalamus and cerebellum in the control calf (No. 141), where euthanasia was performed using a captive bolt, lesions were not evident in the control animal.

Table 4.14 Scores of histological lesions in the respiratory tract.

A. Upper respiratory tract

Calf Number	Mucosal infiltration	Lymphocytic hyperplasia	Focal nasal haemorrhage	Epithelial metaplasia	TOTAL SCORE
340	5	5	4		14
301	3		4	2	9
306			3		3
332		2	2		4
324	0	2	1		3
334		3	1		4
335			1		1
329		2	1		3
309	1				1
320					0
141	1				1
Total	10	14	17	2	43

B. Lower respiratory tract

Calf Number	Peribronchial lymphoid hyperplasia	Bronchial exudate and cellular debris	Increased alveolar septal cellularity	Parasitic granulomas	TOTAL SCORE
340		1			1
301					0
306	1				1
332		1			1
324					0
334	1				1
335					0
329	0	1			1
309	2	1	1		4
320	1	1			2
141				3	3
Total	5	5	1	3	14

(i) The respiratory system.

The lesions observed in the respiratory tract were scored according to severity, and a summary of the results is presented in Table 4.14

The lesions in the upper respiratory tract, particularly the nasal cavity, are much more frequent than the lesions in the lower respiratory tract and are also more clearly related to challenge. The main lesion was the presence of focal haemorrhage at the sites of lymphocytic follicles in the nasal cavity (Figure 4.8). This occurred at regular intervals within the epithelium and submucosa around lymphocytic follicles, where it was sometimes associated with fibrin exudation. The circular appearance of these lesions (seen grossly) was based on the extravasation of erythrocytes in the "shoulder" areas around lymphocytic follicles where the epithelium was seen to invaginate slightly toward the follicle. In the four unvaccinated, challenged calves, these lesions were moderate to severe and widespread, whereas they were only mild in the four vaccinated calves, and absent in the two in-contacts and the control.

Infiltration of the nasal mucosa by inflammatory cells was a feature in two of the challenged calves. Sections of the anterior nasal cavity (turbinate area) of calf 340 showed the presence of globular leucocytes in the pseudostratified columnar epithelial layer, and a few lymphocytes in the underlying submucosa. In contrast, sections of the distal nasal cavity (nasopharynx) showed moderate numbers of neutrophils in the epithelium and large numbers of lymphocytes in the lamina propria. In calf 301, the nasal epithelium contained a diffuse infiltration of moderate numbers of neutrophils. In this animal the trachea showed epithelial metaplasia, with plasma cells and lymphocytes present in the submucosa.

The control calf (No.141) showed only mild mucosal infiltration by eosinophils in both the nasal cavity (Figure 4.15) and trachea, yet had significant lung lesions. The area of consolidation in the caudal lobe consisted of a central focus of eosinophils and neutrophils, surrounded by a broad zone of macrophages, and an outer layer of fibrosis. This lesion was typical of a parasitic granuloma due to *Dictyocaulus* spp. In calf 309, neutrophils were present in the lumina of some bronchi, and also within the epithelial lining. Marked peribronchial lymphoid hyperplasia was also seen in this calf. Mild peribronchial lymphoid hyperplasia was seen in a further three cases, the presence of mild bronchial exudate and cellular debris was seen in four cases and mild increased alveolar septal cellularity was seen in one calf. No lung lesions were found in three calves (Nos. 301, 324 and 335).

Table 4.15 Summary of histological lesion scores for each organ system.

Calf Number	Squamous Epithelium	Abomasum	Intestines	Lymph Nodes	Palatine Tonsils	Spleen & Thymus	Nervous System	Respiratory System	Other Organs	TOTAL SCORES	Group Means
340	12	15	15	10	6		2	15	2	77	
301	24	0	15	10	5		0	9	4	67	
306	15	2	12	20	2		2	4	5	62	
332	19	5	13	27	6	8	0	5	1	84	
											73
324	4	4	18	1	6		4	3	2	42	
334	0	5	5	6	-		3	5	1	25	
335	0	3	4	9	2		1	1	1	21	
329	5	0	13	8	0		0	4	1	31	
											30
309	1	1	6	0	0		1	5	6	20	
320	4	7	3	1	0		0	2	0	17	
141	0	5	2	0	0		2	4	6	19	
											19
TOTALS	84	47	106	92	27	8	15	57	29	465	

(j) Other organs

In the liver, multiple small perivascular foci of mononuclear inflammatory cells were present in seven calves. In three calves (Nos. 306, 334 and 335) these inflammatory foci were mild and in four calves (Nos. 301, 309, 324 and 141) they were moderate. The inflammatory cells were mainly lymphocytes, although in calf 141 eosinophils were also present. As well as multiple accumulations of lymphocytes around blood vessels, calf 309 had one large lymphoid aggregation under the capsule. The liver lesions in both these latter calves were consistent with migrating parasitic larvae. In addition to the perivascular foci in calf 301, the Kupffer cells in the sinusoids were more prominent than normal. The histological scores allocated to the liver lesions did not follow any pattern.

Sarcocysts were found in the myocardium in small numbers in calf 332, and in large numbers in calf 309. Moderate numbers were also found in the masseter muscle in calf 141. Moderate endocardial haemorrhage was observed in calf 306.

The adrenal gland from calf 306 had a wider than normal cortex, which invaginated into the medulla. This diffuse cortical hyperplasia (involving the zona fasciculata) may have been the result of stress.

(k) Summary of histological lesions.

A summary of the total lesion scores is presented in Table 4.15.

The four unvaccinated, challenged calves had the highest lesion scores, with a group mean of 73 (range 62-84), while the mean score of the four vaccinated calves was 30 (range 21-42), almost 60% less. A one-way analysis of variance showed that the difference between these two groups was statistically significant ($p = 0.0007$).

The two unvaccinated, in-contact calves, and the control, had a mean of 19 (range 17-21), 72% less than the mean of the first group of challenged (unvaccinated) animals. A one-way analysis of variance showed that the difference between these two groups was statistically significant ($p = 0.0001$).

The four vaccinates were then compared with the unvaccinated in-contacts, plus the control. The difference between these two groups was found to be approaching significance ($p = 0.0981$).

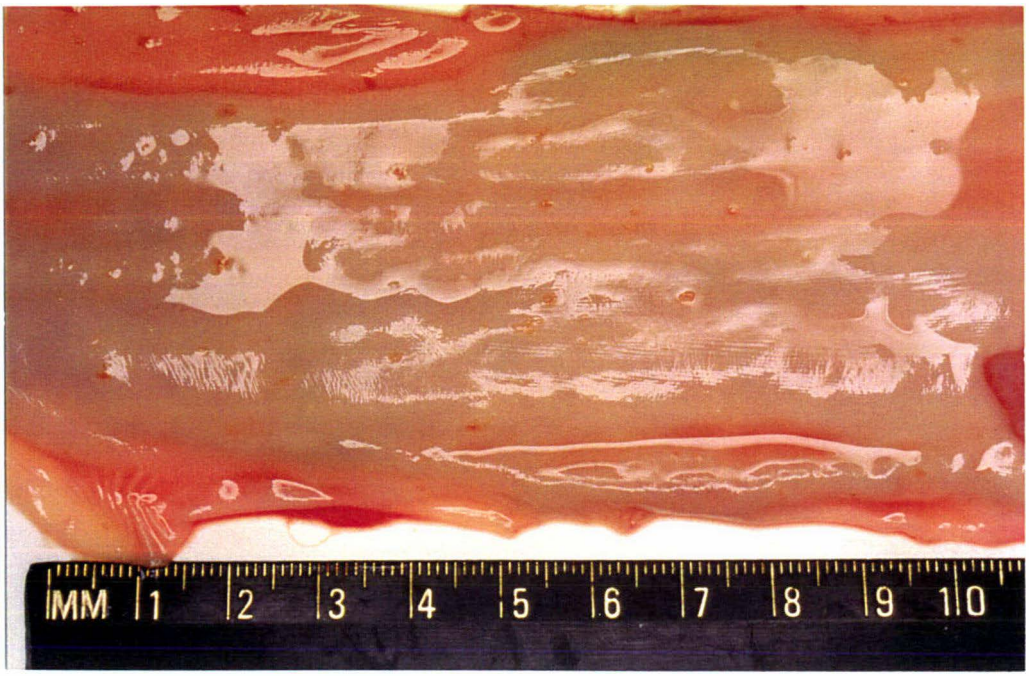


Figure 4.5 Oesophagus, calf 301.
Small erosions (1-2 mm diam.) are scattered throughout the length of the oesophagus.

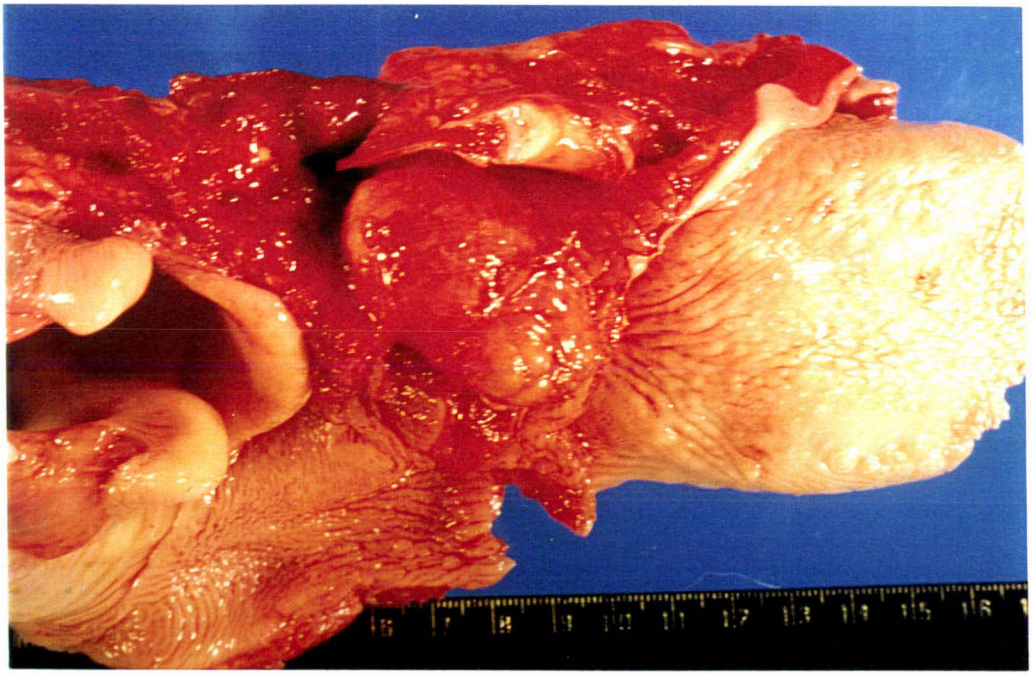


Figure 4.6 Palatine tonsil, calf 332.
The left palatine tonsil is enlarged, measuring 50 mm in length.

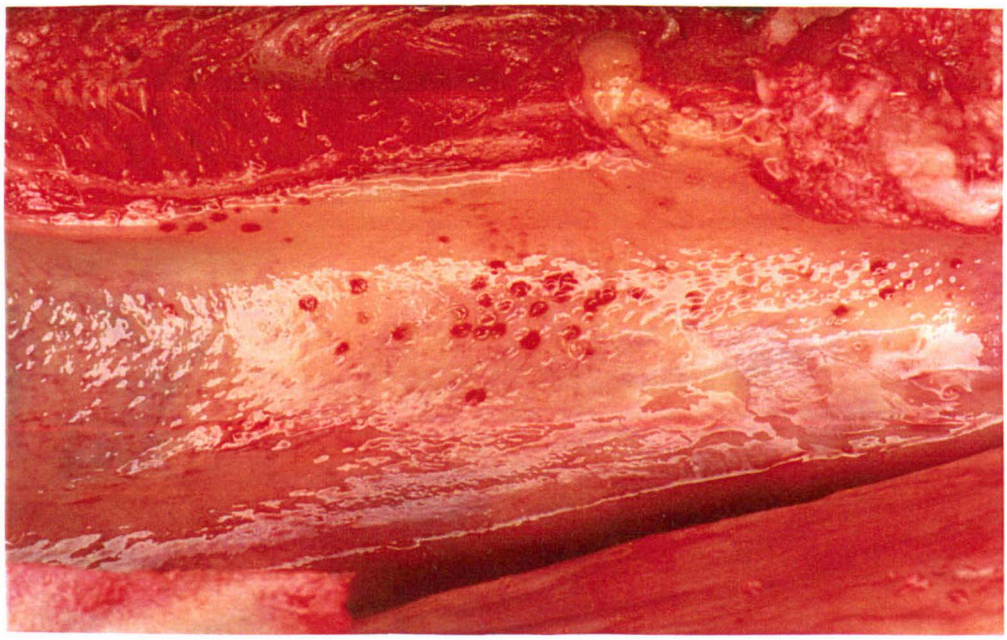


Figure 4.7 Nasal cavity, calf 340 **3x**
Multifocal, small (1-2 mm diam.) and circular haemorrhages in the epithelium.

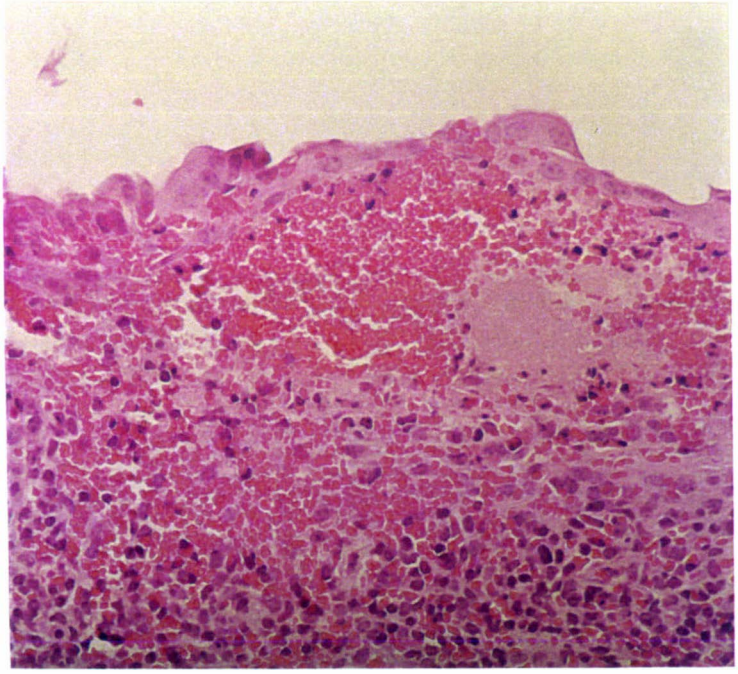


Figure 4.8 Nasal epithelium, calf 301. **150x**
A focus of haemorrhage, hyperaemia and fibrin exudation at the site of a lymphoid follicle.

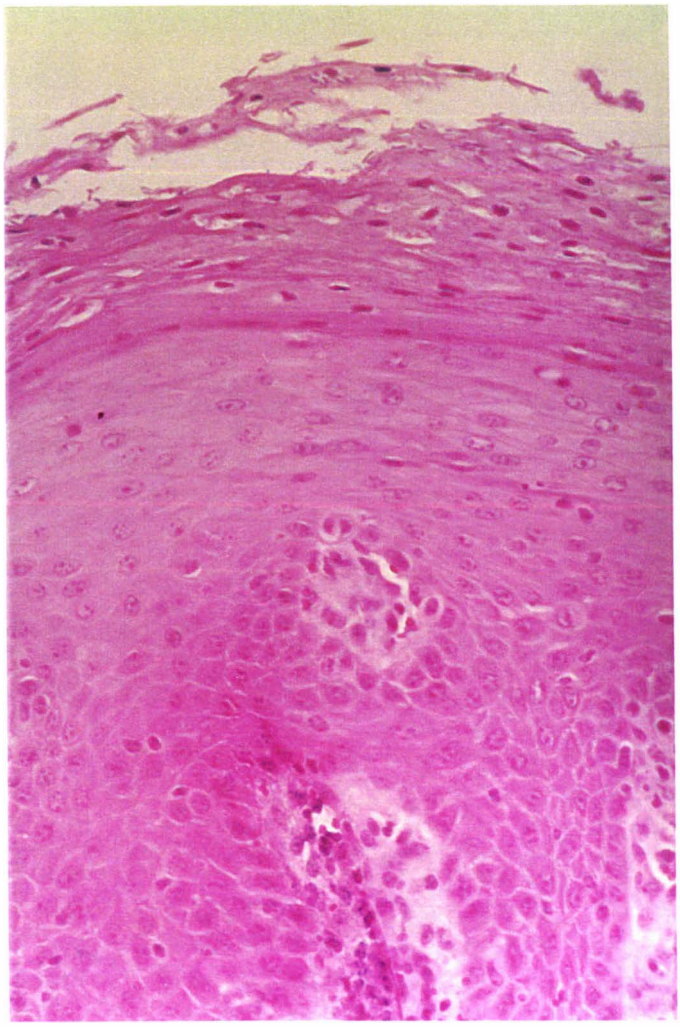


Figure 4.9 Hard palate, challenged calf (No.340). **150x**
An erosive lesion in the stratum corneum, showing parakeratosis and disruption of the superficial epithelium. There is mild neutrophil infiltration into an underlying dermal peg.

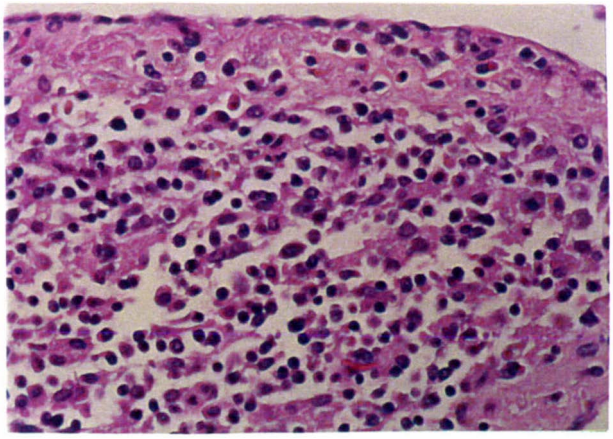


Figure 4.10 Peyer's patch, ileum, from challenged calf (No. 301). **150x**
There is moderate depletion of lymphocytes and reduced mitotic activity.

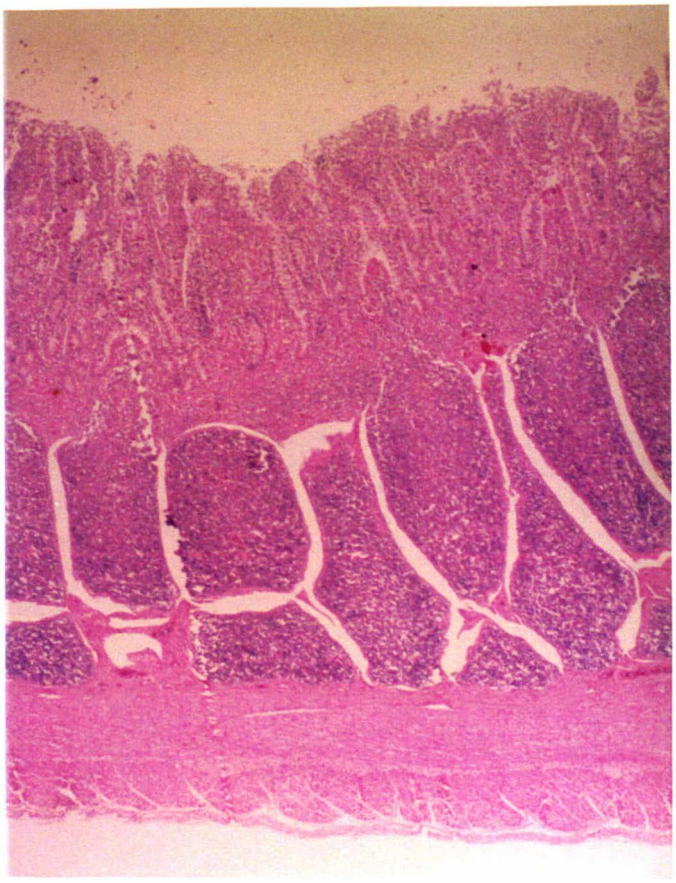


Figure 4.11 Ileum, control calf (No. 141) **x15**
This shows moderate mucosal infiltration by inflammatory cells, mostly eosinophils. Peyer's patches are packed with lymphocytes.

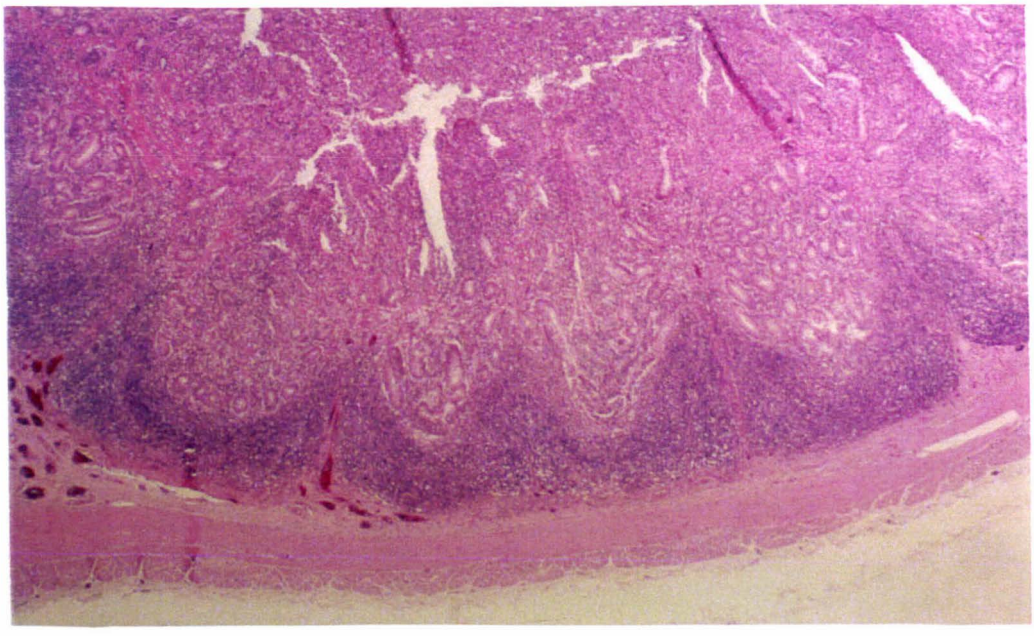


Figure 4.12. Ileum, challenged calf (No.306). **x15**
There is severe cryptal prolapse into sites of depleted Peyer's patches.

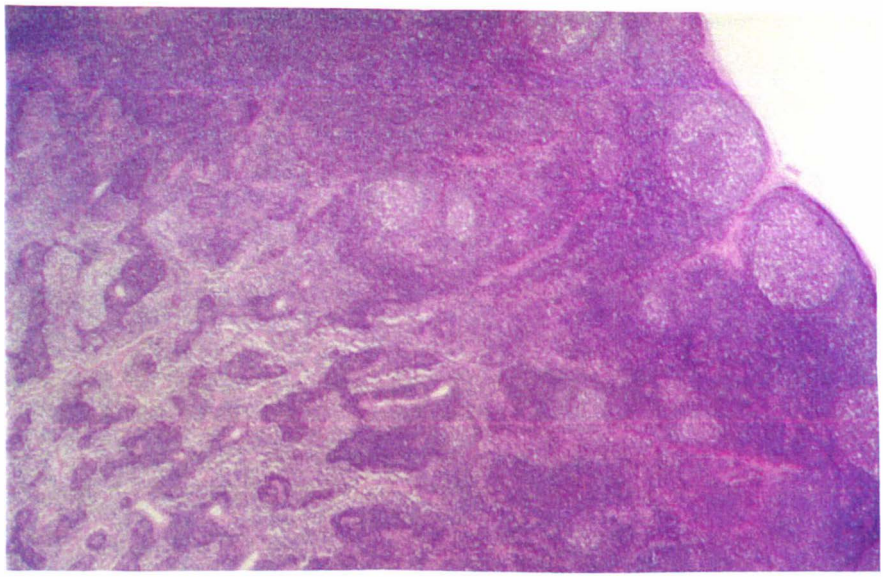


Figure 4.13 Mandibular Lymph Node, control calf (No. 141). x15
Germinal centres (g) are clearly delineated from the paracortical areas (p) and the medullary sinuses (m).

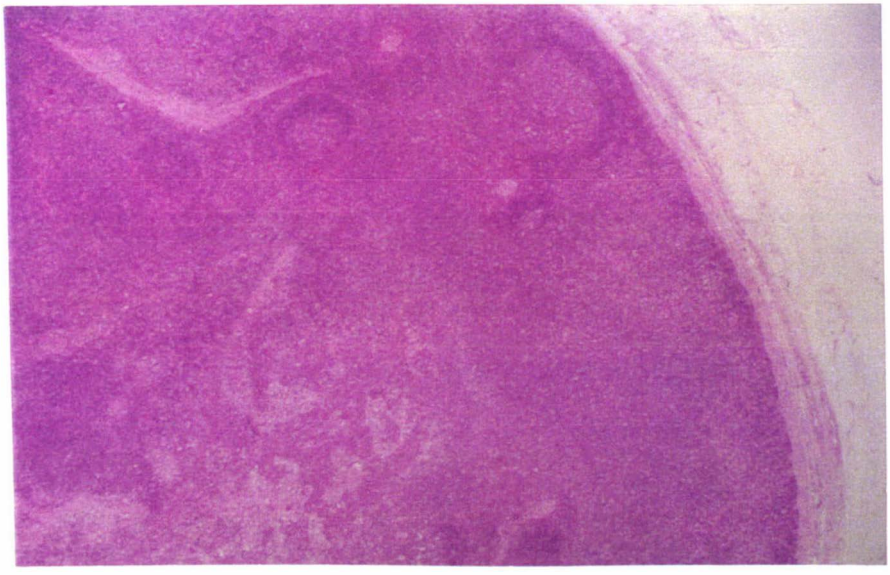


Figure 4.14 Mandibular Lymph Node, vaccinated and challenged calf (No. 324). x15
Large numbers of lymphocytes are present, especially in the paracortical areas, in this enlarged lymph node.

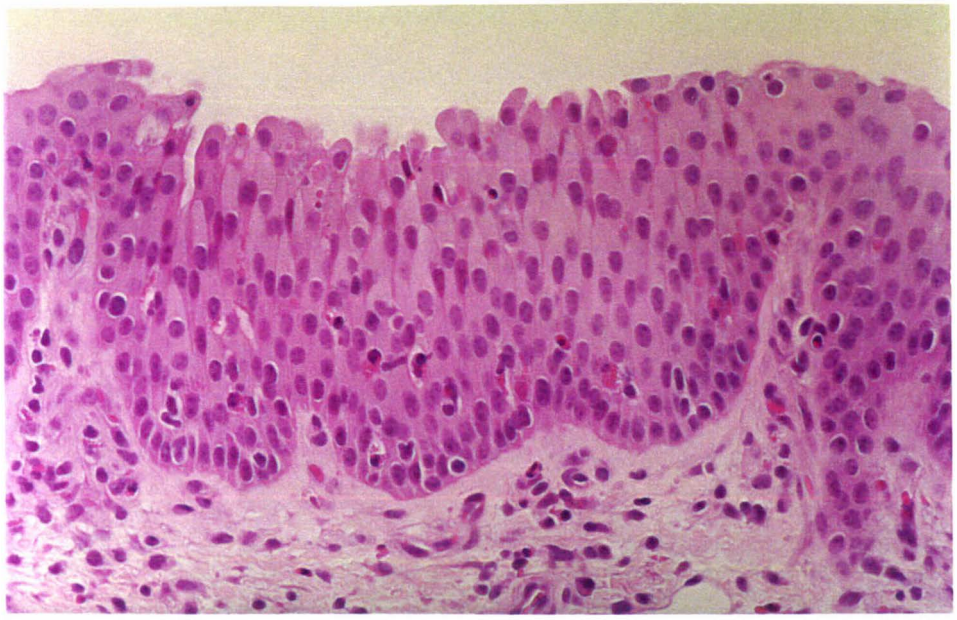


Figure 4.15 Anterior nasal cavity, control calf (No. 141). **x300**
There is mild mucosal infiltration by inflammatory cells. A few eosinophils can be seen in the epithelium, and some plasma cells in the submucosa.

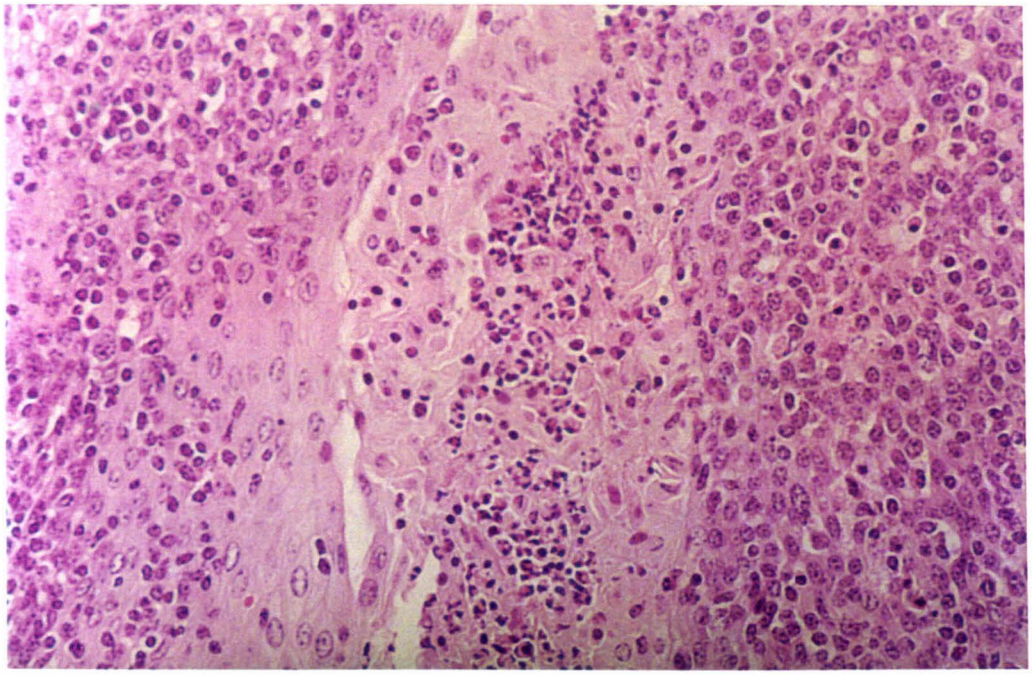


Figure 4.16 Palatine Tonsils, challenged calf (No. 301). **x150**
This tonsillar duct contains a large amount of debris, mainly necrotic neutrophils (n).

4.3.6 Parasitology results

Faecal egg counts were determined for the 10 calves in the transmission experiment, but not the the control animal. Strongylid eggs were found in all but one animal (No. 309), with a mean of 490 eggs per gram (epg) and a range of 0-950 epg (Appendix 4.7).

An abomasal worm count was carried out in 5 calves, with means of 120 for *Haemonchus spp* (range of 0-400), 6,060 for *Ostertagia* spp (range 2,800-12,900) and 9,800 for *Trichostrongylus* spp (range 3,300-18,000). No 4th stage larvae were found. The mean total worm count in the abomasum was 15,980 (range 6,100-24,000).

A worm count of the small intestine was carried out in four calves, with means of 75 for *Nematodirus spp* (range 0-200), 4,575 for *Cooperia spp* (range 2,800-7,000), and 25 for 4th stage larvae (range 0-100). This gave a mean total worm count in the small intestine of 4,675 (range 2,900-7,000).

The mean total worm burden (abomasum plus small intestine) was 20,655.

A larval culture was carried out on a combined faecal sample to determine the proportion of nematodes present. The result was 6% *Ostertagia spp,* 7% *Trichuris spp* and 87% *Cooperia spp*.

4.3.7 Microbiology results

Jejunal contents were collected from three calves (Nos. 301, 306 and 324) and no *Salmonella* or *Yersinia* species were isolated on culture.

Pus from the right bronchus of calf 141 was collected. Occasional Gram-positive cocci were seen on microscopy, and culture revealed a scant growth of *Streptomyces* species.

4.3.8 Antigen and Antibody results

(a) The BVD antigen and antibody ELISA tests.

These were used initially to select calves susceptible to BVDV, and also to ensure a negative status before vaccination and prior to entry to the isolation unit at Massey University.

In detail, six weeks before entry to the university (21st April, 1998), 39 weaned calves (Friesian cross Hereford) were tested, and 29 were found negative to both BVD antigen and BVD antibody. (The 10 remaining calves were postitive for BVD antibody). Two weeks later (7th May, 1998) the animals were weighed and 14 susceptible calves (three

extra calves as reserves) were tested again. These remained negative to both the ELISA antigen and antibody BVD tests. [At the same time six calves were selected as vaccinates and a sensitising dose of "Mucobovin" (Merial) administered]. Three weeks later (28th May, 1998), 7 susceptible and 4 vaccinated animals were selected for the experiment, and tested again. This was now one week before entry into isolation. All 11 calves remained negative for antigen, 10 were negative for antibody, and one was suspicious (No. 141). An SNT was carried out on the sample fom this calf, and the result was negative. [A booster dose of "Mucobovin" was given to the four vaccinates at this time, and the group were provided hay and pellets with limited grass]. These 11 calves were transported to Massey University on the 4th June and settled into two isolation wards.

(b) The SNT

This test was used to measure the level of BVD antibody throughout the experiment, starting at the farm with tests at times of vaccination, followed by tests at the university before and after challenge with virus 47 (Table 4.16 and Figures 4.17 & 4.18)

The titres were expressed as Log $_2$, starting with < 1:2 as zero, 1:2 as 1, 1:4 as 2, 1:8 as 3, 1:16 as 4, etc.

Table 4.16 Antibody levels by SNT in transmission experiment.

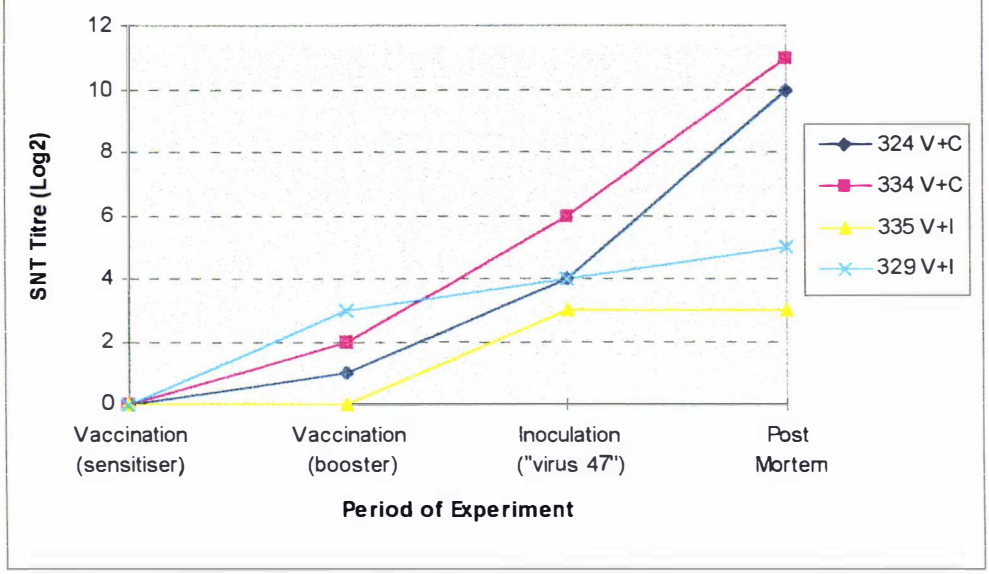
		Titre (Log 2)			
Calf Number	Vaccination (sensitiser)	Vaccination (booster)	Inoculation ("virus 47")	Interim SNT	Post Mortem
340	0	0	0	0	2
301	0	0	0	0	3
306	0	0	0	2	6
332	0	0	0	4	6
324	0	1	4	-	10
334	0	2	6	-	11
335	0	0	3	-	3
329	0	3	4	-	5
309	0	0	0	-	0
320	0	0	0	-	0
141	0	0	-	-	0

(i)The two vaccinates that were also challenged (Nos. 324 and 334) showed an exponential rise in their SNT titres. Following the sensitiser dose of "Mucobovin" their titres were 1:2 and 1:4 respectively, increasing to 1:16 and 1:64 following the booster

vaccination, and finally reaching 1:1024 and 1:2048 after challenge with "virus 47" (Figure 4.17).

(ii)The two vaccinates that remained in-contact only (Nos. 329 and 335) responded to vaccination, reaching titres of 1:16 and 1:8 respectively at the time when the others were challenged. Two weeks later calf 329 had increased by one increment only, to 1:32, while calf 335 remained unchanged at 1:8 (Figure 4.17).

Figure 4.17 Antibody responses in Vaccinated Group

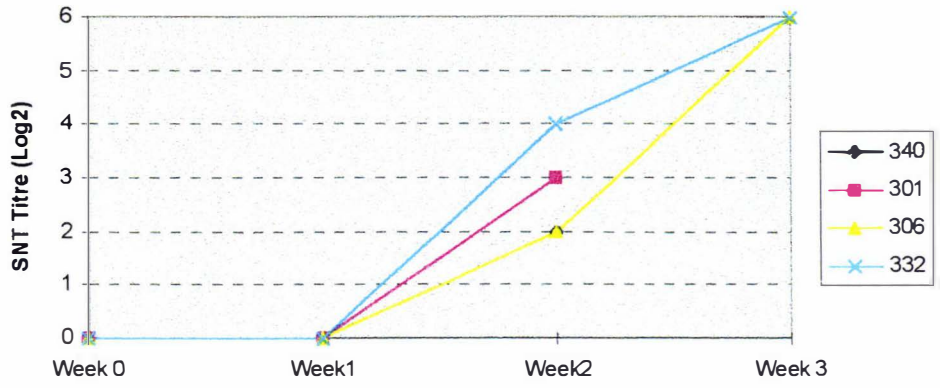


(iii)The four unvaccinated, challenged calves (Nos. 340, 301, 306 and 332) were all negative for antibody pre-inoculation. Two of these (Nos. 340 and 301) were tested 10 days later and still found to be negative, but by 15 days, at necropsy, they had titres of 1:4 and 1:8 respectively. The other two calves (Nos. 306 and 332) were positive at 1:2 and 1:4 respectively at an interim test 14 days after inoculation, and by the time of necropsy (21 days) titres in these two calves had both risen to 1:64 (Figure 4.18).

(iv)The in-contact calves (309 and 320) remained negative for antibody by the SNT throughout the experiment.

(v)The control calf (No. 141) was negative at necropsy, the day after arrival at the university.

Figure 4.18 Antibody in Unvaccinated, Challenged Group



4.3.9 Virus isolation and antigen detection.

(a) Buffy coat.

Forty seven samples of buffy coat from 11 calves (outlined in Table 4.2) were found negative in the BVD antigen ELISA test. These samples had been treated with detergent during the test, so were no longer suitable for any virus isolation proceedures.

(b) Serum.

Twenty serum samples from 10 calves were negative for virus isolation.

(c) Tissue.

Eleven samples of spleen and mandibular lymph node were negative for virus isolation.

4.4 DISCUSSION

4.4.1 Comparison with other experimental infections.

The pathological lesions seen in this experiment were mild and showed similarities to two previous studies in which calves were exposed to NCP Type 1 BVDV isolates. For example, the characteristic lesions in one such study included acute to subacute tracheitis, mild entero-colitis, petechial haemorrhages of mesenteric lymph nodes, with mild follicular depletion, and paracortical lymphocytic hyperplasia (Wilhelmson *et al.*, 1990). However, the original experiments with the New York-1 strain, a Type 1 BVDV, resulted in oral ulceration as the only lesion seen (Baker *et al.*, 1954), while a more recent exposure to this strain caused more severe disease (Marshall *et al.*, 1998). One

calf died, with haemorrhages and a very low platelet concentration (3×10^9 / L), as well as showing fibrinoid necrosis of blood vessels, a lesion not seen in the present study.

The severe lesions described by Bolin and Ridpath (1992) in experimental infections with Type 2 isolates were not a feature of this study. For example, haemorrhages on the serosal and mucosal surfaces of the intestine and splenic capsule were not seen, and petechial haemorrhages in lymph nodes were minor, as was atrophy of splenic white pulp. As with Type 2 strains, however, atrophy of Peyer's patches was a common feature in the present study, with six calves showing depletion of lymphocytes.
In a study by Marshall *et al.* (1996), comparing the distribution of two antigenically distinct Type 2 isolates in SPF calves, microscopic lesions were limited to the lymphoid tissues and GIT. Lymphoid depletion was evident in Peyer's patches and the thymus of some infected calves. Intestinal crypt epithelial cell necrosis, largely confined to the jejunum, was common to both infected groups. Focal areas of necrosis were observed in squamous epithelial cells of the tonsil and ruminal mucosa in some infected calves. High concentrations of BVDV were recovered from the bone marrow of all infected calves, and platelet counts were significantly reduced in all calves during infection. These lesions were similar to those seen in the present study, with the exception of thymic atrophy. Overall, disease with these Type 2 strains was mild, which is unusual, but may have been related to the use of SPF calves, thus eliminating the contribution of opportunist pathogens.

In two further studies (Copari *et al.*, 1989; Copari *et al.*, 1990), 15 of 25 young calves that were experimentally infected with Type 2 strains of BVDV developed severe thrombocytopenia ($<5 \times 10^9$ platelets / L). Thirteen of these calves also developed widespread haemorrhages (eg gingiva, tongue, subcutaneously such as hock, mesentery of small intestine, serosa of jejunum, subdural in the brain, etc). In the present study, only one of 47 platelet tests was below the reference level (200×10^9 platelets / L) and this suggests that the strain used was not a Type 2 BVDV.

The titre of virus used in this experiment was low (10^4 $TCID_{50}$) when compared with other experiments (eg. 10^7 $TCID_{50}$; Marshall *et al.*, 1996).

In summary, it is not possible to firmly catagorise the virus strain on the basis of the pathological consequences of the experimental infection. Nevertheless, the findings obtained here do indicate that the virus used was most likely to be of the Type 1 group, and not related to the NY-1 strain. Sequencing of the genome using PCR techniques would be needed to establish the exact phylogeny. Such studies have identified only the Type 1 strains in New Zealand to date (Vilcek *et al.*, 1998).

4.4.2 BVD infection status.

(a) Infection status based on pathological findings.

In the study of the field outbreak (Chapter 3), it was found that three lesions were common to all BVD infected calves; i.e. erosive lesions in the squamous epithelium of the upper alimentary tract, degenerative and hyperplastic lesions in lymphoid tissues, and focal necrotising enteric lesions. The presence of these lesions in the calves in the transmission experiment was used to gauge their BVD infection status. (Table 4.17).

Table 4.17 BVD Infection Status based on pathological findings.

Calf Number	Erosive Lesion Score	Lymphoid Lesion Score	Enteric Lesion Score	BVD Infection Status
340	8	16	9	+ve
301	10	15	9	+ve
306	5	22	10	+ve
332	4	41	6	+ve
324	1	7	9	+ve
334	0	6	2	-ve
335	0	11	0	-ve
329	2	8	3	+ve
309	0	0	0	-ve
320	1	1	0	-ve
141	0	0	0	-ve

Erosions and/or ulcers.

The four unvaccinated, challenged calves were all confirmed histologically to have erosions and/or ulcers. This group had the greatest number of erosions at necropsy (Table 4.9), as well as the highest histological scores for confirmed erosions and/or ulceration (Tables 4.10 and 4.15).

Erosions were not detected in the two vaccinated, challenged calves (Table 4.10). One of these calves (No. 324) did have very superficial, multifocal oesophageal lesions at necropsy, but on histological examination these were found to be areas of hyperkeratosis and parakeratosis rather than erosions. A healed erosion was also seen on the tongue of this calf at necropsy. It was concluded this animal should be classified as 'suspicious' and was assigned a score of 1 (Table 4.17).

Of the two vaccinated, in-contact calves, one had two confirmed oesophageal erosions. At necropsy, healed erosions were also observed on the hard palate and tongue of this calf. The other calf had no observable lesions.

Of the two unvaccinated, in-contact calves, one (No. 320) did have a large ulcer on a rumen pillar, but it was advanced and organised, indicating that it was more likely to be a physical trauma rather than due to BVDV infection. This animal was classified as 'suspicious' and given a score of 1 (Table 4.17). The other calf had no observable lesions.

The unvaccinated, not in-contact calf showed no erosive lesions in the upper alimentary tract.

Lymphoid lesions.

The combined histological scores of the lymph nodes, palatine tonsils, spleen and thymus showed that all but the two unvaccinated in-contact calves, and the control, had lesions (Table 4.15). The mild inactivity in the germinal centres in lymph nodes in calf 320 was probably not significant, but this animal was classified as suspicious and given a score of 1 (Table 4.17).

Enteritis.

In the study of the disease outbreak (Chapter 3) it was concluded that the findings of crypt necrosis, cryptal prolapse and lesions in Peyer's patches (depletion, necrosis or mucosal inflammation) are intestinal lesions that may be diagnostic for BVD in the absence of rinderpest (Barker *et al.*, 1993). These lesions were used as measures of enteric infection in the transmission experiment.

Crypt necrosis or cryptal prolapse were seen in all the six challenged calves, but were not seen in any of the other calves.

The four unvaccinated, challenged calves and two of the vaccinated calves, one challenged and one in-contact, had lesions in the Peyer's patches. These were lymphocyte depletion and reduced activity, lesions that clearly indicate the presence of BVDV, which has an affinity for Peyer's patches (Barker *et al.*, 1993). Calf 329 (a

vaccinated, in-contact animal) showed lymphocyte depletion, the presence of macrophages, and also some necrosis in germinal centres. This provided evidence that this calf was infected with BVDV by contact with the inoculated animals. This conclusion was supported by the presence of subcutaneous petechial haemorrhages and a 50% drop in platelet concentration at necropsy.

Using a pathological basis for diagnosis, it was concluded that the four unvaccinated, challenged calves (Nos. 340, 301, 306 and 332) as well as one vaccinated, challenged calf (No.324) and one vaccinated, in-contact calf (No. 329) fulfilled the criteria for a classification as "positive for BVD disease" (Table 4.17). This was further supported by the total histological scores for these calves (Table 4.15), where these six animals are seen to have the highest total scores.

(b) The infection status as defined by serology and virology.

All four unvaccinated, challenged calves showed a rise in BVD antibody in the SNT at two weeks after inoculation with virus 47 [from <1:2 to 1:4 (two calves), 1:8 (one calf) and 1:16 (one calf)]. Two were retested a week later, and their titres had increased by four-fold and sixteen-fold (Table 4.16). This confirmed BVDV infection of this group.

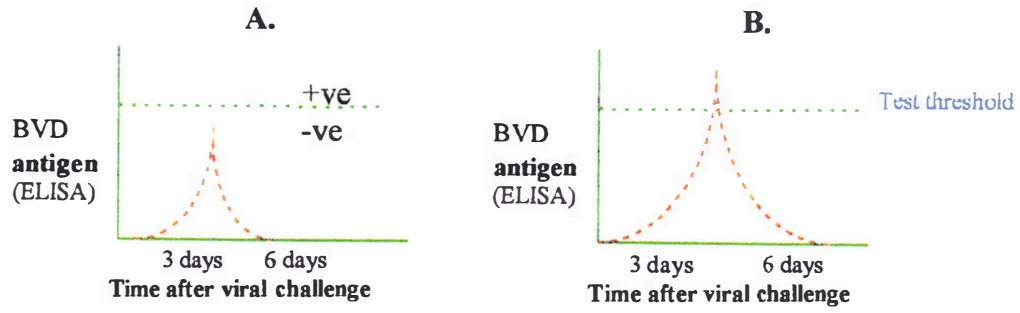
In the vaccinated group, two calves were challenged, and after two weeks their titres rose dramatically from 1:16 and 1:64, to 1:1024 and 1:2048 respectively. This confirms BVDV infection in these two calves. The titres of the two vaccinated, in-contact calves showed little change, one remaining the same at 1:8, while the other increased from 1:16 to 1:32 (Table 4.16).

The two unvaccinated, in-contact calves remained negative for antibody by the SNT throughout the experiment. However, BVDV infection that occurred at <14 days before necropsy would not have allowed sufficient time for detectable antibody production. In this study, antibody response was not detectable by SNT until 15 days after infection. Other workers have shown that in acute infection, neutralising antibodies appear at 14 days and slowly increase to reach a maximum at 10-12 weeks post infection (Howard *et al.*, 1989). Thus BVDV infection of in-contact animals could have occurred, despite the negative antibody results.

No antigen was detected in buffy coat samples collected during the experiment.

Howard *et al.* (1989) found there is only limited recovery of virus from blood (and nasal secretions) during the first 3-10 days p.i. in acute infections, and by 14 days virus cannot be isolated. These negative results could indicate that the ELISA test was not sensitive enough to detect the rather low level of virus present in white blood cells (Figure 4.19, A), or that antigen only surpassed the cut-off point for a short period of time, and this did not coincide with the time of sampling (Figure 4.19, B).

Figure 4.19 Proposals for negative antigen results.



When levels of BVD antigen are very low, as seen early in the course of an acute infection, they may not be detected by the ELISA test used at the CAHL (GW Horner, personal communication).

Virus isolation from serum samples was negative. However, virus levels may have been quite low in the serum in these acutely infected animals, in contrast to PI calves or those with MD. In addition, the samples had been stored at -20 ^{0}C, as they had originally been intended for antibody testing only. Furthermore, the time interval between collection and virus isolation was almost a year, so the likelihood of survival of the virus would be very low, as viability at this temperature has only been demonstrated up to 6 months (Ssentengo, 1978).

Virus isolation from tissues was also negative. Although samples had been adequately stored at -70 ^{0}C, they were collected at necropsy, 12-21 days after viral challenge, so with this time delay, virus isolation would not be expected in these acute infections.

The negative virus isolation results raises the possibility that BVDV infection did not occur. However, this is negated by the rise in BVD antibody titres demonstrated in all

the six challenged calves, and is further confirmed by the presence of typical histological lesions at necropsy.

4.4.3 Effect of vaccination.

The vaccination titres of the four calves injected twice with 'Mucobovin' (Merial) were relatively low (1:8, 1:16, 1:16 and 1:64) when measured seventeen days after their booster dose. In a survey of neutralising antibody levels in New Zealand cattle (Perez *et al.*, 1994), a titre of 1:16 was found to be at the trough of the distribution curve. In this survey and also in the present transmission experiment, titres were measured using NADL, a CP strain of BVDV, in the test. Previous work carried out in our laboratory on cattle vaccinated twice with 'Mucobovin' (Merial) showed that when NADL was used as the neutralising virus, the titres obtained were low, similar to the levels described above (unpublished). With Bovax, a CP strain of New Zealand origin, titres were similarly low. The titres were much higher in these animals when the vaccine strains, New York (NY) and Aveyronite-4 (AV-4), were used in the test. With six other New Zealand strains, titres were intermediate between NADL / Bovax and the homologous strains, NY and AV-4.

It is possible that the NCP virus used in the transmission experiment (virus 47) was antigenically different from the vaccine strains, which would mean that the high level of antibodies to NY and AV-4 may not be cross-protective. The histological lesions did not align virus 47 with the NY strain (a NCP, bovine strain of American origin) and the AV-4 strain is a NCP ovine strain of French origin, which is unlikely to be antigenically similar to the challenge virus.

Although all platelet concentrations remained within the reference range, following inoculation, platelet counts in the unvaccinated, challenged group (four calves), dropped progressively at 3 and 6 days p.i., then returned to pre-inoculation levels at 11 days. The opposite trend occurred in the vaccinated, challenged group (two calves), with a rise over the first two sampling periods, and a return to pre inoculation levels by 11 days. This could indicate that vaccination had a protective effect on platelet concentration, and possibly even enhanced the response. However, statistical evaluation of these small groups is not meaningful, so the significance of these variations is uncertain.

With the in-contact calves (two vaccinated and two not), minor variations in platelet counts were noted through to day 11, after which a marked fall occurred in the

unvaccinated in-contact calves, which is consistent with infection shortly before necropsy. Again, groups of two animals presented problems for statistical evaluation.

Comparison of group means for total WBC concentration also followed a trend, despite the fact that only 2 of 47 tests were outside the reference range. There was an immediate fall in total WBCs by 3 days p.i. in the unvaccinated, challenged group, while all other groups had risen at this point. This same picture was mimicked by the lymphocyte concentration, which indicated that it was this fraction of the WBCs that were being affected by the BVDV. This lymphopenia was quite temporary, with a rise in concentration by 11days, and a return to pre infection levels at necropsy. Transient leucopenia is a feature of acute infection with BVDV, with a decrease of both B and T lymphocytes by 3-7 days, and recovery to normal by 11-17 days post infection (Bolin *et al.*, 1985). These results suggest that vaccination was able to shorten this period of transient lymphopenia.

The histological lesion scores of the four vaccinated calves were significantly less than those of the four unvaccinated, challenged calves ($p = 0.0007$). The organ systems which made the greatest contribution to these lower lesion scores were the upper alimentary tract, the lymphoid system, and the respiratory system. In particular, there was a marked reduction in both the number and score for erosions and ulceration in the UAT, considerably reduced lesions in the germinal centres of lymph nodes, and lower lesion scores for focal nasal haemorrhage in the URT.

The total histological lesion scores of two of the vaccinated calves (one challenged and one in-contact) were very low, and in the same order as the two unvaccinated, in-contact calves and the control. This suggested a protective effect of the vaccine. The other vaccinated, challenged calf had a higher score and at necropsy, multifocal, superficial oesophageal lesions were observed, although histological examination confirmed that these were areas of keratosis, not erosions. A healed erosion was also found on the tongue of this calf. It is possible that the vaccination of this challenged animal reduced the effect of the inoculated virus by preventing overt oesophageal erosions from developing. The second vaccinated, in-contact calf showed some lesions suggestive of BVDV infection. However, once again oesophageal erosions were minor, and erosions seen in the hard palate and tongue were healed at necropsy, suggesting a benefit from vaccination.

The weight gain of the vaccinated calves was found to be greater than that of the unvaccinated, challenged animals (674 gm/day vs 86 gm/day). However, this was not found to be statistically significant ($p = 0.1165$). One of the unvaccinated, challenged calves (No. 340) showed a dramatic weight loss after inoculation, markedly affecting the mean of this group. A trial using greater numbers would therefore be needed to firmly establish a protective effect on growth rate for vaccination in the face of BVDV challenge.

In summary, the most significant effect of vaccination was to reduce the disease severity (histological lesion scores) by 60%. This partial protection may indicate that the challenge strain was antigenically divergent from the vaccine strains.

4.4.4 Pathogenesis of BVDV lesions.

(a) Lymph node lesions.

The mandibular lymph nodes were enlarged in five calves, with measurements being similar to the dimensions of adult glands (30-40 mm long and 20-30 mm wide; Sisson and Grossman, 1964). Paracortical hyperplasia was a feature of both the unvaccinated, challenged calves, as well as the vaccinated calves (challenged or in-contact). However, it was not seen in the unvaccinated, in-contact calves, nor in the control. Assuming that the paracortex is the site where T-lymphocytes accumulate in the lymph node, this picture indicates that antigen stimulation (challenge or vaccination) has resulted in the migration of T-lymphocytes to the lymph nodes from either the peripheral circulation or thymus.

Depletion of lymphocytes in the germinal centres was seen in five of the six challenged calves, but not in those unchallenged. This indicates that the BVDV attacks B-lymphocytes in the germinal centres, which would in turn result in a reduced antibody response in infected animals. BVDV infection has been shown to suppress interferon production, impair lymphocyte function, monocyte proliferation and chemotaxis, humoral antibody production, neutrophil function and bacterial clearance, as reviewed by Barker *et al.* (1993). Inactivity of germinal centres (reduced mitosis) was significant in seven calves, but no clear pattern emerged, except that the unvaccinated, in-contact

calves were unaffected, as was one of the vaccinated calves (No. 324) which may suggest a degree of protection had been afforded to B-lymphocytes.

(b)Abomasal lesions

Mucosal infiltration by inflammatory cells, especially eosinophils and globular leucocytes, together with hyperplasia of lymphoid follicles, indicated that gastric parasitism may have been an important complication. This was confirmed by the parasitology results, showing the abomasal worm burden was high (*Trichostrongylus* and *Ostertagia* spp).

The total abomasal score for one of the challenged calves was high. All of the six different lesions measured were found to be present in this calf. This was probably the result of a combined BVDV and *Ostertagia* infection, and may provide the basis for this animal's dramatic weight loss (10 kg) following inoculation. The total abomasal scores for each of the other calves did not appear to be related to viral challenge.

(d) Lesions in the intestine

The main lesion seen was mucosal infiltration by inflammatory cells, particularly eosinophils, plasma cells and lymphocytes. Because this lesion was common to all calves, and the eosinophils predominated, severe nematode parasitism was likely to be the underlying cause. This was confirmed by the presence of nematodes in crypts and lumina in many sections, and parasitic granulomas in two calves. Parasitology results confirmed a high worm burden in the small intestine, almost exclusively *Cooperia* spp. The mean total worm burden for the abomasum plus the small intestine was high, especially when the drenching history of the mob at the farm is taken into account (six weekly endectocide). In addition, strongylid eggs were found in the faeces of all but one calf, with a mean level that was close to the point of clinical significance. With minor evidence of inhibited 4th stage larvae, this count was likely be a good reflection of the level of worm burden. A larval culture on a combined faecal sample confirmed the large population of *Cooperia* spp present (87%).

In conclusion, widespread parasitic infection in the GIT made it difficult to assign some lesions to a viral cause. For this reason only those intesinal lesions considered characteristic of BVDV infection (crypt necrosis and cryptal prolapse) were used to diagnose infection status.

In the field outbreak, crypt lesions were only seen in mucosal disease cases, while in the transmission experiment, all six challenged calves developed lesions (crypt necrosis or cryptal prolapse). These were also reported in another experiment where only NCP virus was used (Marshall *et al.*, 1998). It is concluded that these distinctive lesions are not exclusive to mucosal disease, but occur in acute infection also.

Mucosal congestion of the intestine was only seen in four of eleven calves, which contrasted with the calves from the field outbreak where mucosal congestion was a frequently observed lesion, especially in the ileum. The method of euthanasia chosen for this experiment (barbiturate anaesthesia and exsangiunation) suggested that the extensive congestion seen in the field outbreak was possibly a result of use of the barbiturate. However, because the transmission experiment resulted in a simple acute infection rather than MD, the ileal congestion seen in the field outbreak calves could still be a feature of mucosal disease.

(e) Lesions in the respiratory tract.

Focal nasal haemorrhages were found in the challenged and vaccinated calves but not in the unvaccinated, in-contact calves, or the control calf. This could mean that they occurred only in those animals stimulated by antigen (virus or vaccine) and may represent increased function of the lymphocytic nodules, with dramatically increased vascularity. Thus the lesions seen could represent an extravasation of erythrocytes in the vicinity of highly active lymphocytic follicles rather than acute petechial haemorrhages. The circular form of the lesions would further support this hypothesis.

The increased vascularisation and lymphoid hyperplasia is likely to have taken place over the period between infection or vaccination and necropsy.

The nasal lesions described here have not been reported in the natural disease. The intra-nasal route of infection cannot be held as the cause in all cases, as they were also found in the vaccinated, in-contact calves. It is possible that they have been overlooked by other workers, as full examination of the nasal cavity is not a simple exercise. It is also possible that these focal nasal haemorrhages were a feature of this particular viral isolate.

In the lower respiratory tract, the lesion scores for the unvaccinated in-contact calves, plus the control, contributed 64% of the grand total for all calves, indicating that the viral challenge may not be an important causal factor for these lesions.

4.5 SUMMARY

I. The pestivirus used in the transmission experiment (virus 47) caused mild disease in weaned calves, with lesions similar to experiments where Type I isolates (other than N.Y.-1) had been used. The changes in haematological indices were mild, with no evidence of the severe thrombocytopenia and widespread haemorrhages characteristic of Type 2 isolates.

II. There was evidence of spread of pestiviral infection to a vaccinated, in-contact calf, based on the presence of gross and histological lesions typical of BVDV infection in this animal.

III. Vaccination with a locally available, inactivated pestiviral vaccine ("Mucobovin", Merial) resulted in a 60% reduction in total histological lesion score. In particular, the vaccine markedly reduced the number and severity of erosions and ulcers in the upper alimentary tract, was protective against lymphocyte depletion in germinal centres of lymph nodes and reduced the severity of focal nasal haemorrhage in the upper respiratory tract. The vaccine also reversed the fall in platelet concentration, delayed the onset of lymphopenia, and reduced the weight loss associated with BVDV challenge.

IV. The partial protection afforded by the BVDV vaccine ("Mucobovin") may indicate that the challenge virus (strain 47) was antigenically heterologous to the vaccine strains NY-1 and AV- 4, or simply that this is the level of protection that can be expected with this vaccine against homologous virus.

V. A serological diagnosis of BVDV infection in this experiment was made by demonstrating a greater than fourfold rise in titre of BVD antibody in all challenged calves.

VI. Virus isolation was negative in this experiment, but this is probably a common occurrence in early acute infections where virus levels are low.

VII. Lymph node enlargement was found to be associated with paracortical hyperplasia, and was a feature of both challenged and vaccinated calves.

VIII. Lesions due to nematode parasites were common in the gastro-intestinal tract and lungs. The synergistic affect of BVDV and *Ostertagia* spp on abomasal function was demonstrated in one challenged calf that showed dramatic weight loss after inoculation.

IX. Mucosal congestion in the small intestine was not common, which may have been due to the method of euthanasia, or because this lesion is not a feature of acute infection.

X. The finding of lymphocytic rhinitis, with haemorrhages at follicular sites, in challenged and vaccinated calves, may be associated with the common finding of nasal discharge soon after infection (widespread at six days post inoculation in this study).

XI. The use of small numbers of animals (2-4) in each treatment group in this study presented problems with statistical analysis. However, the differences in histological score between the "unvaccinated, challenged" group and the "vaccinated" group or the "unvaccinated, in-contacts plus control animal" were highly statistically significant.

XII. Although this experiment suggests that the challenge virus is a Type 1 strain with the expected level of virulence, definitive classification would require molecular comparison with known, defined, BVDV strains.

CHAPTER FIVE

GENERAL DISCUSSION

The investigation into this case of high mortality (48%) in young calves, indicated that it was due to widespread mucosal disease (MD). Since MD results when PI (carrier) animals are super-infected by a CP biotype of an antigenically similar strain of bovine pestivirus, this outbreak is likely to have started with the birth of an unusually large cohort of PI calves, well in excess of the expected prevalence of 0.5 to 2% (Houe, 1999). This can be put into perspective when considered on a herd basis. The prevalence of herds with BVDV antibody is very high, in the order of 70% to 100% in most countries, indicating that the majority of herds have experienced past or present infection. From investigation of a few in-depth studies of whole herds, it was concluded that about half of those herds that are seropositive are actively infected, as indicated by the presence of PI animals. It is conceivable that some herds could have larger numbers of PI animals purely by chance, due to high infection rates during the risk period (45-125 days) during pregnancy. This would occur if there were a large number of serologically negative pregnant cows exposed about the same time. Although this may be the simplest explanation for outbreaks where high numbers of PI animals are found, there is also recent evidence that some strains of BVDV are associated with certain clinical syndromes (Houe, 1996), and it may be possible that the NCP strain isolated from the field outbreak had an enhanced ability to produce PI animals.

The possibility that a highly pathogenic strain of BVDV was involved in this outbreak could not be ruled out after inital investigations. Although five cases were confirmed to be MD, six other calves investigated were confirmed as acutely infected, showing widespread and severe lesions. Of the calves that developed clinical signs, or were found to be BVD antigen positive by the field veterinarian, many had died at an earlier age than would be expected for MD (< 6 months). Experimental transmission of a NCP isolate from the outbreak was carried out in an attempt to investigate its pathogenicity. Typing of the virus using molecular techniques (PCR) would have been valuable at this point to rule out the possibility of Type 2 BVDV, but unfortunately, this was beyond the financial scope of the project. Results from the transmission experiment, however, did

indicate that the virus was probably not a Type 2 strain and the disease pattern observed was that expected from Type 1 strains, including spread to in-contact calves.

The total dose of BVDV used per calf in the transmission experiment was 3.2×10^4 $TCID_{50}$, administered both orally and intranasally. Seroconversion followed administration of this dose, and lesions indicating BVDV infection were found. The minimum dose of BVDV required to elicit a detectable antibody response was found in one study to be 2 mLs of a suspension containing $10^{3.3}$ $TCID_{50}$ per ml, when administered by the intranasal route (Cook *et al.*, 1990). Since the main method of transmission is by direct nose-to-nose contact, this finding could serve as a guide to the minimum infectious dose of BVDV and equates to approximately 4×10^3 $TCID_{50}$.

The present study has shown that a presumptive diagnosis of bovine pestiviral disease can be made following careful ante- and post-mortem examination. The combined presence of erosive lesions in the upper alimentary tract (buccal cavity and oesophagus), catarrhal enteritis, and lesions in the lymphoid tissues (e.g. lymph node enlargement, haemorrhage or necrosis, and inflammation of Peyer's patches) are indicative of BVDV infection. Confirmation of these lesions can be made histologically, and observation of some distinctive bowel lesions (crypt necrosis of glands of Lieburkuhn, or herniation of crypts into the submucosal site of depleted Peyer's patches) is considered diagnostic for pestiviral infection in New Zealand. A major shortcoming of this work was the inability to confirm that the lesions seen were definitely caused by the BVD virus. The use of immunohistochemistry would have demonstrated the presence of virus in the tissue sections and confirmed the association between virus and lesion. A large bank of tissues were stored appropriately for this purpose, and are available for a future study to describe the location of viral antigens in infected tissues.

Lesions in the respiratory tract were a feature in this study. Half of the calves from the field outbreak showed respiratory lesions, which was probably indicative of the immunosuppressive effect of the BVD virus. In the transmission experiment there was widespread nasal discharge 6 days post-inoculation and rhinitis was observed in challenged and vaccinated calves. A study of the distribution of BVDV following acute infection (Bruschke *et al.*, 1998) indicated that the virus first replicates in the nasal mucosa, then can be found at high titres in the tonsil. It is therefore concluded that the

presence of a nasal discharge may indicate acute BVDV infection, and that clinicians should include BVDV infection in the differential diagnosis of upper respiratory disease.

The rhinitis observed was lymphocytic in nature, with haemorrhages at follicular sites. This was only seen in animals stimulated by antigen, either virus or vaccine, which may indicate that these were sites of immunological reaction.

The widespread presence of GIT parasitism in calves in both the field outbreak and transmission experiment reflected the usual autumn peak for nematode infections observed in these areas of New Zealand. However, it was difficult to discriminate between the generalised cell-mediated response to the virus and the nematodes. Dependence on the farmer's anthelmintic program (six-weekly endectocide) for nematode control in calves used for the transmission experiment was a shortcoming of the transmission study. This could have been remedied by treatment with a different anthelmintic (e.g. oxfendazole) prior to entry to the isolation facility.

Virological diagnosis of BVDV infections is not always a simple matter, especially with acute infection, due to the limited time that virus is present. In the transmission experiment reported here, virus was unable to be isolated from the calves. However a significant rise in antibody titre between acute and convalescent samples was demonstrated. This serological method of diagnosis should be used more often to confirm BVDV infections. Some examples of this include investigations into "repeat breeding", where mated heifers or cows continue to return to service at a normal cycle length, and "late returns" where females return to heat more than 30 days after mating. Investigation of these early signs of reproductive failure by collection of blood samples 3 to 4 weeks apart could reveal if BVDV is the cause. Investigation beyond 3 months (e.g. at time of pregnancy testing) would be too late, as a rise in titre will no longer be detectable, and high antibody levels at this time only indicate prior contact with BVDV, which is common in the population.

Diagnosis of abortion due to BVDV is also difficult. If antibody or antigen can be detected in the aborted foetus, then intrauterine infection with BVDV is proven (Houe, 1996). Distinctive lesions in the aborted foetus may also confirm foetal infection (Jubb and Huxtable, 1993). However, abortion is not always the result of direct viral invasion

of the foetus, but can be secondary to damage to the maternal placenta through disruption of its vascular supply (Brownlie, 1990). In experimental infections, abortions exceeded 30%, but recovery of virus was infrequent (Brownlie *et al.*, 1986) suggesting that placental damage may have been the major cause of foetal death. With acute infection, there is only limited recovery of virus from the blood for 3-10 days post-infection, while antibody is measurable by 2 weeks and rises to a peak by 10-12 weeks (Howard *et al.*, 1989). Consequently, in the absence of virus, the diagnosis of BVDV abortion in the cow (or heifer) may only be possible through demonstrating a rise in antibody titre during this 3 month period post infection. Even when it is demonstrated, however, this only indicates that infection occurred around the time of abortion, so the direct detection of virus in foetus or placenta remains the most definitive means of diagnosis.

Survival time for the BVD virus reduces with rising temperature. If whole blood is refrigerated (4 ^{0}C) the virus was shown to be stable for 35 days (Tanaka *et al.*, 1968) while at room temperature (20 ^{0}C) BVDV could be isolated for at least 5 days after collection from PI animals (Rae *et al.*, 1987). In the present study, EDTA blood samples collected for antigen ELISA testing were sent to the CAHL in Lower Hutt on the day of collection. One sample was inadvertently left in the refrigerator for 9 days after collection before being processed and sent to CAHL, where it was found negative for antigen. The lesions at necropsy of this animal were typical of BVD and a follow-up spleen sample was positive for antigen. This emphasises that the ELISA test on whole blood samples should be carried out as soon as possible after collection, especially in acute infection cases. It is recommended that testing be carried out within 5 days of collection, and that an ice pack be included with the posted sample.

Eradication of BVDV is being attempted in some areas in the northern hemisphere where prevalence is low (e.g. Finland) or where the disease has only recently been found (e.g. Shetland Islands) or where vaccination has not been practiced (e.g. Scandinavian countries). Eradication is based on the premise that the infectivity of pestiviruses outside the host is of short duration, and that the most important means of transmission is direct contact with a PI animal. The programs depend on identification and removal of PI animals, followed by the implementation of measures that prevent reintroduction of infection. Two of these countries (Finland and Shetland) are nearing

eradication, but difficulties have been reported with the final removal of virus (Synge *et al.*, 1999; Nuotio *et al.*, 1999). It may be that other species are important in the transmission of BVDV. The virus has been isolated from other ruminants, including sheep and goats, and has also been transmitted to sheep and back to cattle via PI animals (Paton *et al.*,1997). About one tenth of farmed deer in New Zealand have antibodies to BVDV (Motha, 2000), while in Norway, reindeer have a higher seroprevalence than cattle and sheep (Loken, 1995), raising the possibility of a reservoir among free-living ruminants. Phylogenic analysis of pestiviruses from domestic and wild ruminants indicate that Type 1 strains occur world-wide in many species (Becher *et al.*, 1997).

The farming of cattle in New Zealand contrasts markedly with that in the northern hemisphere, requiring a different approach to disease control. The main differences are a seasonal calving pattern, outdoor or pastoral farming (which is often extensive in the beef industry) and much larger herds, especially in the dairy industry. BVDV infection is endemic (as indicated by a level of 60% seropositivity in both beef and dairy cattle), vaccines available are unproven (only provisionally licensed), and the number of herds showing evidence of infection is high (the proportion that are seropositive is in the order of 85%).

It has been suggested that it would be advantageous to identify herds naive to BVDV and raise their immune status to the virus by vaccination in order to protect them from severe manifestations of disease should BVDV be inadvertently acquired (Wilks, 1994). Identification of these naive herds in the dairy industry could be achieved by using the indirect antibody ELISA test ("Svanovir", Sweden) on bulk milk samples (Niskanen, 1991; Niskanen, 1993). For beef herds, blood testing 5 animals aged 6 to 18 months, as described by Houe (1991), would identify the herds without antibody. The naive herds could then be vaccinated to raise their immune status.

It has also been recommended that the immune status of replacement heifers at 12 months of age should be ensured (by either vaccination or exposure to PI animals) before the breeding season (Golo, 1989). This is an excellent aim, but its execution remains a problem. The use of PI animals to immunise the rising yearlings is feasible, but the risk of infecting pregnant cows or heifers is too high for this method to be used. Vaccination has been shown to reduce the level of PI animals in a herd, but not to

eradicate the infection, so vaccination on its own may give some control but will be unable to completely eradicate the infection. The crucial factor in the control program is the identification and elimination of PI animals, as they are pivotal to the maintenance of infection, including spread to the next generation. The detection of PI animals as young calves would be beneficial to the farmer. The presence of colostral antibody to BVDV makes diagnosis from whole blood impossible in the calf less than 8 months old. However, a novel method of detecting BVD antigen in skin by immunohistochemical means (Thur *et al.*, 1996) could be used on skin samples at any age. An ear-marking tool could be used both to collect a sample and identify the calf sampled. If such a technique proved to be as accurate as that reported (more accurate than ELISA tests and equivalent results to virus isolation) then results would be valuable in indicating herds with active infection, and the removal of the PI animals would dramatically reduce the risk of infection in these groups of young animals. However the removal of PI animals would render these groups susceptible to the disease, especially when heifers (or bulls) entered the main herd. Consequently, immunisation of the calves would also be required to protect them. The recommended time for initiating this would be about 8 months, when maternally derived antibody has waned. One method would be the use of autogenous vaccines, made from the serum of the PI animals detected on the farm. The eradication of Enzootic Bovine Leucaemia would need to be complete before this method could be used, and the risk of infecting pregnant cattle remains high. The other option is the use of a commercial vaccine.

The vaccine used in my transmission experiment was shown to be only partially protective, with a 60% reduction in total histological lesion score, and spread of infection to one of two vaccinated, in-contact calves. This may have been due to antigenic heterology between the challenge strain and the vaccine strains. It is now considered that the antigenic differences between bovine pestiviruses is far greater and of more significance than was originally realised (Bolin, 1995; Bolin, 1996). The production of a vaccine containing a number of different strains, with high levels of antigen, would seem advisable. A group at the Elizabeth McCarther Agricultural Institute, Camden, New South Wales, Australia, are testing such a multiple strain vaccine for its ability to protect the foetus (personal communication, A. D. Shannon, 1998), and this may be a sound approach.

Only two BVDV vaccines ("Bovidec", C-Vet, Britain and "Resvac 4", Pfizer, America) have been shown to prevent transplacental infection, which is a necessary requirement

to prevent the production of a PI animal. With the inactivated vaccine ("Bovidec)" this result was achieved by using an additional booster dose in 60% of the vaccinates (i.e. 3 doses at monthly intervals). With the live vaccine ("Resvac 4") one dose was not completely successful (83.3%) but it was probable that a booster dose may have ensured complete protection. It would be useful if all BVD vaccines available in New Zealand provided evidence for protection of the foetus. An investigation into the effect of an additional booster dose is recommended.

In summary, a combined program of removal of PI animals (using immunohisto chemical diagnosis) and vaccination (using a vaccine proven to protect the foetus) in rising yearling calves (heifers and bulls) would ensure both freedom from and immunity to bovine pestivirus disease before the mating period. If this was repeated annually for the time it took to turn the herd over (only 7 years in some dairy herds) then the whole herd would be clear of infection by this time. This type of program could be adopted at a farm, district, or national level.

Economic losses resulting from BVDV infection are significant throughout the world. When converted to the cost per cow, or per calving, the range is N.Z.$ 20-114. The mean annual losses for Denmark in 1992, before control measures were introduced, was N.Z.$ 40 per calving. This would equate to N.Z.$ 6000 per herd of 150 cows per year. Reproduction losses due to BVDV in N.Z. have been estimated at N.Z.$ 30-40 per cow. In a severe case in a New Zealand beef herd, where all manifestations of the disease occurred, the loss was N.Z.$ 110 per expected calving, which was close to the level of losses reported due to Type 2 strains in North America (N.Z.$ 114 per calving). These are significantly high losses that indicate the need for some form of disease control. Further benefits of BVD control would be a reduction in other diseases which arise due to the immuno-suppressive effect of the virus, and also the avoidance of confusion with several exotic diseases which have a similar clinical presentation.

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APPENDICES

Appendix 2.1 Reagents used for cell culture

1. Acetone fixative (20%)

25ml Acetone
25mg Casein Hydrolysate

Made up in 100ml distilled water, and stored at room temperature.

2. Amino acid solution (BRL)

MEM Non Essential Amino Acid Solution (1%).
Stored at 4 ^{0}C.

3. Antibiotic-Trypsin-Versene (ATV)

0.5g Trypsin (Difco, 1:250)
0.2g Versene (EDTA sequestric acid)
8.0g NaCl
0.4g KCl
1.0g Dextrose
0.58g $NaHCO_3$
$2x10^5$ IU Penicillin
100mg Streptomycin
0.02g Phenol Red

Made up to 1L with distilled water. Sterilised by filtration. Adjusted to pH 7.2. Stored at - 20^{0}C before use, then kept at 4^{0}C.

4. Eagles minimum essential media (E-MEM)

9.7g MEM (Sigma cell culture)
2.2g $NaHCO_3$

Made up to 1L with distilled water. Sterilised by filtration. Adjusted to pH 7.2-7.4. Stored at 4^{0}C.

5. Growth media (GM)

Bovine embryonic lung (Belu) cell culture
MEM was the basic growth medium to which was added :-
15% Equine Serum (ES)
1% Antibiotic (PSK)
1% Vitamins (BRL)
10% Tryptose Phosphate Broth (TPB)

Stored at 4^{0}C.

Madin Darby bovine kidney (MDBK) cell culture

MEM was the basic growth medium to which was added :-
10% Equine Serum (ES)
1% Antibiotic (PSK)

Stored at 4^0C.

6. Maintenance Medium (MM)

As for growth medium for both cell lines but with the ES reduced to 5%.

7. Penicillin / Streptomycin / Kanamycin (PSK)

$1x10^6$ IU Penicillin
1g Streptomycin
1g Kanamycin

Made up in 100ml of PBS. Sterilised by filtration. Stored at -20^0C before use, then kept at 4^0C.

8. Phosphate buffered saline (PBS)

8.0g NaCl
0.2g KCl
1.15g Na_2HPO_4
0.2g KH_2PO_4

Made up to 1L with distilled water. Sterilised by autoclaving. Adjusted to pH 7.2-7.4. Ca^{++} and Mg^{++} free. Stored at 4^0C.

9. Tryptose phosphate broth (TPB)

20g Tryptose
2g Dextrose
5g NaCl
2.5g Na_2HPO_4

Made up to 1L with distilled water. Sterilised by autoclaving Adjusted to pH 7.3. Stored at 4^0C.

10. Trypan blue solution

0.2g Trypan blue powder
100ml PBS

Stored at 4^0C before use, then kept at room temperature.

Appendix 3.1 Virology samples collected from field outbreak calves (continued over).

CALF NUMBER	**96**	**12**	**28**	**26**	**39**	**66**	**43**	**15**	**67**	**24**	**47**	**73**
Date of Necropsy	1.5.97	2.5.97	5.5.97	7.5.97	7.5.97	8.5.97	8.5.97	9.5.97	9.5.97	24.4.97	-	-
BLOOD COMPONENTS												
1.Buffy Coat-Heparin	+	+	+	+	+	+	+	+	+	-	+	-
2.Buffy Coat-EDTA	+	-	+	+	+	+	+	+	+	-	+	+
3.Serum-Ab	a.	a. & b.	a. & b.	a. & b.	a. & b.	a. & b.	a. & b.	a. & b.	a. & b.	a.	a.	a.
4.Spleen (back up)	+	-	+	+	+	+	+	+	+	-	-	-
INTESTINAL SAMPLES												
Mouth Ulcers	Gum	Gum		H.P.				Tongue	Tongue			
Oesophagus	Ulcer	Ulcer			Ulcer							
Reticulum												
Rumen			Ulcer									
Omasum												
Abomasum	+	+	+			+						
Duodenum												
Jejunum						+			+			
Ileum	+	+	P.p.						+			
LYMPH NODES												
Hepatic									+			
Mandibular				+		+	+					
Prescapular								+				
Mesenteric	+					+			+			
Ileal		+	+	+					+			
Mediastinal					+							
Retropharyngeal			+	+								
PANCREAS	+	+				+		+	+			

Appendix 3.1 Cont'd

CALF NUMBER	96	12	28	26	39	66	43	15	67	24	47	73
GUT CONTENTS	Faeces				S.I.							
SALIVA							+	+	+			
NERVOUS TISSUE												
Cerebrum		+										
Spinal Cord		+										
BLOOD RELATED												
Thymus	+	+	+	+			+	+	+			
Spleen	+	+	+	+	+	+	+	+	+			
Thyroid	+	+	+									
Tonsil				+	+	+		+	+			
Liver	+	+	+	+			+	+	+			
Lung	+	+	+	+	+		+	+	+			
Kidney	+	+	+	+			+		+			
URINE								+	+			

LEGEND
a = first or "acute" sample
b = second or "convalescent" sample
H.P. = hard palate
S.I. = small intestine
P.p. = Peyer's patch

FOOTNOTE
Serum samples stored at -20 ^{0}C, while all other samples stored at - 70^{0}C.

Appendix 4.1 Pestivirus Transmission Experiment - Samples collected at necropsy (continued over).

CALF NUMBER	***141***	***340***	***301***	***335***	***324***	***309***	***332***	***306***	***334***	***329***	***320***
Date of Necropsy	5.6.98	24.6.98	24.6.98	26.6.98	26.6.98	29.6.98	30.6.98	1.7.98	1.7.98	2.7.98	3.7.98
BLOOD COMPONENTS											
1.Buffy Coat-Heparin	V	V	V	V	V	V	V	V	V	V	V
2.Whole Blood-EDTA	CP	CP	CP	CP	CP	CP	CP	CP	CP	CP	CP
3.Serum	S	S	S	S	S	S	S	S	S	S	S
ALIMENTARY SYSTEM											
Mouth	H, V	H, V	H	H	H	H	H, V	H, V	H	H, V	H
Oesophagus	H	H, V	H, V	V	H	H, V	H,V	H, V	H	H, V	H
Reticulum	H, V		H	H	H	H	H	H	H	H	H
Rumen	H, V		H	H	H	H	H	H	H	H	H
Omasum	H		H	H	H	H	H	H		H	
Abomasum	H	H	H	H	H	H, V	H, V	H	H	H, V	H
Duodenum	H, V	H, V	H	H, V	H	H	H	H	H	H	V
Pancreas	H, V	H, V			H, V	H, V	H, V	H, V	H	H	H, V
Jejunum	H	H	H	H, V	H, V	H	H	H	H, V	H	
Ileum	H, V	H	H, V	H, V	H	H	H	H	H, V	H	H
Peyer's patch	H	H	H	H, V	H, V	H, V	H	H, V		H	H
Caecum	H	H	H	H	H	H, V		H		H	
Colon	H, V	H		H	H		H	H		H	H
Rectum	H,V				H			H			H
LYMPH NODES											
Sub Mandibular		H	H, V	H, V	H, V	H, V	H	H, V	H	H, V	
Prescapular								H			
Mesenteric	H	H		H	H	H, V		H,V	V	H, V	V
Ileal	H						H				
Mediastinal	H, V		H	H, V	V	H, V					H, V
Retropharyngeal	H,V						H			H, V	
Abomasal						H, V					
Hepatic							H				

Appendix 4.1 Continued

CALF NUMBER	*141*	*340*	*301*	*335*	*324*	*309*	*332*	*306*	*334*	*329*	*320*
OTHER LYMPHOID											
Thymus	H	H, V		H		H, V	H, V	H, V	H, V	H, V	H, V
Spleen	H, V	H, V	V	H, V		H, V	H, V	H, V	H, V	H, V	H, V
Tonsil	H, V	H, V	H, V	H	H	H	H, V	H, V	H, V	H, V	H, V
NERVOUS SYSTEM											
Brain	H		H	H		H	H	H	H		V
Spinal Cord	H		H	H	H	H	H	H	H		
Eye	H										
RESPIRATORY											
Nasal Epithelium	H	H	H		H	V	H	H	H	H	
Nasopharynx			H			H					
Turbinate		H	H								
Trachea	H										
LUNG	H	H, V	H, V	H, V	H	H	H, V	H, V	H, V	H, V	H
LIVER	H, V	H, V	H, V	H, V	H, V	H, V	H, V	H, V	H, V	H, V	V
KIDNEY	H, V	H	H, V	H, V	H, V	H, V	H, V	H, V	H, V	H, V	V
OTHER											
Myocardium	H					H	H	H			
Ovary/uterus							H, V			H, V	H, V
Adrenal						H	H	H			
Thyroid									H		
ABOMASAL CONTENTS							P	P	P	P	P
S. I. CONTENTS			B		B			P, B	P	P	P
SALIVA		V	V			V	V			V	
URINE											

Legend:- Sample Identification --- H = Histology; V = Virology; S = Serology; CP = Clinical Pathology; B = Bacteriology; P = Parasitology

Appendix 4.2 Weight in Kg's of calves in transmission experiment.

Calf No.	Farm **7/05/98**	Farm **28/05/98**	Massey - # **11/06/98**	Massey - P.M. **24/06-3/07/98**
340	169.0	184.0	196.0	186.0
301	194.5	213.0	223.0	225.0
306	187.0	198.0	202.0	213.0
332	164.5	185.0	187.0	195.0
Chall. mean	178.8	195.0	202.0	204.8
324	174.0	188.0	189.0	204.0
334	168.5	186.0	196.0	208.0
335	174.5	195.0	200.0	210.0
329	148.5	162.0	165.0	174.0
Vacc.mean	166.4	182.8	187.5	199.0
309	190.0	208.0	212.0	-
320	188.0	207.0	216.0	-
141	192.5	210.0	-	-
Mean (All)	177.4	194.2	198.6	201.9
Wt. Gain		**800 gm/day**	**314 gm/day**	**186 gm/day**

Legend : P.M. = Post Mortem
= Weighed the day before challenge with "virus 47" (12/06/98)

Appendix 4.3.1 Results of haematological tests on red blood cells.

Calves challenged with BVD virus.

Analyte	RBC	Hb	Hct [PCV]	MCV	MCH	MCHC	
Ref. Range	5.0-10.0	80-140	0.24-0.40	40-60	14.4-18.6	260-340	
Units	$x10^{12}$/l	g/l [%]	l/l	fl [u3]	pg	g/l	
Calf No.							
- Period							
340-O	7.10	107	0.31	45	15.0	335	N
340-I	7.03	104	0.30	44	14.7	338	N
340-II	6.87	95	0.29	43	13.8	320	N
340-III	7.20	102	0.32	45	14.1	315	N
340-IV							
306-O	8.38	131	0.38	45	15.6	344H	N
306-I	7.24	110	0.32	45	15.1	340M	N
306-II	7.45	109	0.33	44	14.6	330	N
306-III	6.49	96	0.29	45	14.7	328	N
306-IV	7.87	107	0.36	46	-	-	N
301-O	7.14	117	0.35	49	16.3	331	N
301-I	7.31	116	0.35	46	15.8	343H	N
301-II	7.31	112	0.33	46	15.3	331	N
301-III	6.80	105	0.32	47	15.4	325	A
301-IV							
332-O	8.93	139	0.41H	46	15.5	335	N
332-I	7.62	109	0.34	45	14.3	315	N
332-II	8.48	121	0.38	45	14.2	315	N
332-III	7.14	105	0.32	46	14.7	322	A
332-IV	7.36	107	0.33	46	14.7	-	A
324-O	7.89	117	0.34	43	14.8	342H	N
324-I	7.96	113	0.34	43	14.1	327	N
324-II	8.40	115	0.35	42	13.6L	325	N
324-III	7.48	104	0.31	42	13.9L	330	N
324-IV							
334-O	7.51	121	0.34	46	16.1	346H	N
334-I	7.86	119	0.36	46	15.1	329	N
334-II	7.58	113	0.34	46	14.9	327	N
334-III	7.31	110	0.33	46	15.0	329	N
334-IV	7.33	-	0.32	44	-	-	N
MEAN	7.52	112	0.34	45	14.9	330	
Range	6.49-8.93	95-121	0.29-0.41	42-49	13.6-16.3	315-344	

LEGEND H = High values; M = Marginal values; L = Low values
N = Erythrocytes appear normal
A = Anisocytosis of erythrocytes
= vaccinated against BVDV ('Mucobovin")

Appendix 4.3.2 Results of haematological tests on red blood cells.

Calves not challenged with BVD virus.

Analyte	RBC	Hb	Hct [PCV]	MCV	MCH	MCHC	
Ref. Range	5.0-10.0	80-140	0.24-0.40	40-60	14.4-18.6	260-340	
Units	$x10^{12}$/l	g/l [%]	l/l	fl [u3]	pg	g/l	
Calf No.							
- Period							
335-O	8.41	130	0.37	45	15.4	345H	N
335-I	9.17	136	0.39	44	14.8	340M	N
335-II	8.65	119	0.37	43	13.7L	319	N
335-III	8.10	117	0.34	43	14.4	335	N
335-IV							
329-O	8.62	136	0.39	45	15.7	347H	N
329-I	7.93	120	0.35	45	15.1	336	N
329-II	7.96	119	0.35	45	14.9	333	N
329-III	7.41	111	0.33	45	14.9	332	N
329-IV	8.41	-	0.36	44	-	-	N
309-O	7.86	129	0.37	47	16.4	346H	N
309-I	8.93	142H	0.41M	46	15.9	346H	N
309-II	8.76	135	0.40M	46	15.4	335	N
309-III	8.24	128	0.37	46	15.5	340M	N
309-IV	8.48	137	0.38	45	16.1	350H	N
320-O	7.86	127	0.36	46	16.1	351H	N
320-I	8.27	128	0.37	45	15.4	340M	N
320-II	7.68	119	0.35	46	15.4	337	N
320-III	7.72	116	0.34	45	15.0	332	N
320-IV	7.83	-	0.34	43	-	-	N
141-O	9.28	153H	0.45H	49	16.4	338	N
MEAN	8.28	128	0.37	45	15.4	339	
Range	7.41-9.29	111-153	0.33-0.45	43-49	13.7-16.4	319-350	

LEGEND H = High values; M = Marginal values; L = Low values
N = Erythrocytes appear normal

= vaccinated against BVDV ("Mucobovin")

Appendix 4.4.1 Results of haematological tests on platelets,etc.

Calves challenged with BVD virus.

Analyte	Plat.s	MPV	PCT	PDW		Fibrinogen	Total Solids
Ref. Range	200-600					2.0-7.0	67-85
Units	$x10^9/l$	fl	L/L			g/L	g/L
Calf No.							
- Period							
340-O	391	4.7	0.00183	14.8		3	70
340-I	378	4.5	0.00170	15.5		6	68
340-II	305	4.5	0.00137	15.5	#	2M	67M
340-III	449	4.4	0.00197	13.6		5	71
340-IV							
306-O	429	4.4	0.00188	13.6		4	71
306-I	400	4.5	0.00180	15.5		5	70
306-II	423	4.2	0.00154	16.6		2M	67M
306-III	472	4.2	0.00198	14.2	#	4	67M
306-IV	453	4.3	0.00194	13.9		5	70
301-O	426	4.1	0.00174	14.6		4	72
301-I	407	4.2	0.00170	16.6		4	72
301-II	369	4.2	0.00154	16.6	#	5	72
301-III	372	4.0	0.00148	17.5		4	68
301-IV							
332-O	474	4.6	0.00218	15.2		5	73
332-I	415	4.7	0.00195	14.8		4	67M
332-II	354	4.6	0.00162	15.2		4	67M
332-III	537	4.4	0.00236	15.9		3	64L
332-IV	466	4.6	0.00214	15.2		3	63L
324-O	396	4.4	0.00174	13.6		5	62L
324-I	455	4.5	0.00204	15.5		4	62L
324-II	451	4.5	0.00202	15.5		4	62L
324-III	381	4.5	0.00171	15.5		6	63L
324-IV							
334-O	343	4.8	0.00164	14.5		5	66L
334-I	367	4.7	0.00172	14.8		4	66L
334-II	361	4.7	0.00169	14.8	#	6	66L
334-III	350	4.7	0.00164	14.8		6	67M
334-IV	339	4.6	0.00155	15.2		6	66L

LEGEND Red = calves inoculated with virus 47

= calves vaccinated against BVDV ("Mucobovin")

= clumping of platelets seen in smears

H = high values; M = marginal values; L = low values

Appendix 4.4.2 Results of haematological tests on platelets, etc.

Calves not challenged with BVD virus.

Analyte	Plat.s	MPV	PCT	PDW		Fibrinogen	Total Solids
Ref. Range	200-600					2.0-7.0	67-85
Units	$x10^9/l$	fl	L/L			g/L	g/L
Calf No.							
- Period							
335-O	410	4.4	0.00180	13.6		5	73
335-I	441	4.4	0.00194	15.9		4	74
335-II	441	4.5	0.00198	15.5	#	5	70
335-III	443	4.6	0.00203	15.2		4	69
335-IV							
329-O	436	4.3	0.00187	13.9		3	70
329-I	364	4.3	0.00156	13.9		4	64L
329-II	447	4.3	0.00192	13.9		3	65L
329-III	441	4.4	0.00194	13.6		2	65L
329-IV	204	3.7	0.00075	16.2	#	5	70
309-O	340	4.4	0.00149	15.9		6	71
309-I	345	4.5	0.00155	15.5		4	72
309-II	317	4.5	0.00142	15.5		4	72
309-III	278	4.7	0.00130	14.8	#	2	70
309-IV	193L	6.6	0.00289	15.0		3	71
320-O	390	4.5	0.00175	15.5		2	63L
320-I	404	4.4	0.00177	13.6		3	64L
320-II	449	4.4	0.00197	15.9		5	72
320-III	490	4.6	0.00225	15.2		4	66M
320-IV	358	4.5	0.00161	15.5		2	67M
141-O	462	4.7	0.00217	14.8		6	71

LEGEND Blue numbers = in contact calves
141 = "control calf"
= vaccinated against BVDV ("Mucobovin")
= clumping of platelets seen in smears

H = high values
M = marginal values
L = low values

Appendix 4.5.1 Results of haematological tests on white blood cells.

Calves challenged with BVD virus.

Analyte	W.B.C.	Neut.	Lymp.	Mono.	Eos.	Baso.
Ref. Range x 10^9/L	4.0 - 12.0	0.6 - 4.5	1.8 - 7.5	0.08 - 0.70	0 - 2	0 - 0.2
Calf No.						
-Period						
340-O	10.6	2.6	7.2	0.1	0.7	0
340-I	9.1	3.8	4.7	0.4	0.2	0
340-II	5.4	2.1	2.9	0.3	0.1	0
340-III	8.9	3.8	4.8	0.3	0	0
340-IV						
306-O	12.9 H	2.2	8.3 H	1.4H	0.9	0.1
306-I	5.1	0.92	3.8	0.2	0.2	0.1
306-II	5.2	1.2	3.8	0.2	0.1	0
306-III	7.8	2.0	4.9	0.6	0	0.4H
306-IV	10.4	3.7	5.0	0.7	0.7	0.3H
301-O	11.6	3.6	6.7	0.6	0.2	0.5 H
301-I	8.9	5.6 H	3.6	0.4	0	0
301-II	9.1	4.3	4.7	0.1	0.1	0
301-III	9.8	1.3	7.5M	0.9H	0	0.2M
301-IV						
332-O	12.0	3.3	7.3	0.4	1.2	0.01
332-I	6.9	0.8	5.5	0.1	0.4	0
332-II	8.8	2.4	5.8	0.1	0.4	0.1
332-III	9.2	1.8	6.7	0.5	0	0.3H
332-IV	11.2	2.9	7.7H	0.4	0.1	0.1
324-O	9.2	1.9	6.6	0.3	0.3	0.9 H
324-I	11.6	5.4 H	5.4	0.2	0.5	0.2M
324-II	6.7	2.6	3.3	0.1	0.8	0
324-III	7.2	1.7	5.1	0.2	0.2	0.2M
324-IV						
334-O	8.2	2.6	5.2	0.3	0.2	0
334-I	9.0	1.9	6.6	0.5	0	0.1
334-II	6.1	1.6	4.3	0.1	0.2	0
334-III	8.5	2.0	6.0	0.3	0.2	0.1
334-IV	8.8	2.7	6.0	0.2	0	0

LEGEND = vaccinated against BVDV ("Mucobovin")

Red = calves inoculated with virus 47.

H = high values; M = marginal values; L = low values

Appendix 4.5.2 Results of haematological tests on white blood cells.

Calves not challenged with BVD virus.

Analyte	W.BC.	Neut.	Lymp.	Mono.	Eos.	Baso.
Ref. Range x 10^9/L	4.0 - 12.0	0.6 - 4.5	1.8 - 7.5	0.08 - 0.70	0 - 2	0 - 0.2
Calf No.						
-Period						
335-O	8.7	2.8	4.9	0.3	0.5	0.3 H
335-I	10.3	4.4	5.5	0.3	0.1	0
335-II	8.2	3.9	4.0	0.2	0.3	0
335-III	8.6	3.5	4.6	0.3	0.1	0.2
335-IV						
329-O	11.6	2.3	8.1 H	0.5	0.7	0
329-I	11.7	2.6	8.7H	0.1	0.4	0
329-II	11.6	3.5	7.4	0.4	0.4	0
329-III	11.6	4.2	7.2	0.1	0.1	0
329-IV	11.6	3.5	6.7	0.2	0.8	0.4H
309-O	9.5	1.4	6.5	0.1	1.4	0.1
309-I	13.9H	5.6H	6.7	0.3	1.1	0.3
309-II	10.0	2.0	6.0	0.8	1.1	0.1
309-III	10.8	3.0	5.8	0.1	0.2	0.3H
309-IV	9.7	2.1	6.1	0.4	1.2	0.1
320-O	11.2	2.5	7.2	0.3	1.2	0
320-I	11.1	1.8	8.3H	0.1	0.7	0.2
320-II	9.3	1.1	7.6H	0.2	0.2	0.3H
320-III	9.0	2.0	6.8	0.2	0	0.1
320-IV	10.2	2.6	6.1	0.6	0.8	0.1
141-O	9.8	3.3	5.9	0.6	0.1	0

LEGEND H = High values; M = Marginal values; L = Low values
Blue = in-contact calves
= calves vaccinated against BVDV ("Mucobovin")
141 = "control" calf

Appendix 4.6 Infiltration of the intestinal mucosa (lamina propria) by inflammatory cells.

A. SEVERITY SCORE and CELL TYPE at each SITE

Calf No.	Duodenum	Jejunum	Ileum	Small Int.	Caecum	Colon	Large Int.	Mean Score for Intestine
340	1-EPL	3-EPL	3-EPL				1-EPL	2
301				3-EPL			3-EPL	3
306	2-PEL	3-EPLM	2-EPL				2-PEL	2
332	3-PLE	3-ELP	2-LEP			2-LEP		3
324	4-ELP	4-ELP					2-E	3
334		2-EP	2-EP			2-EP		2
335	3-PE	3-EPL	3-ELP		2-EPL	2-EPL		3
329	3-GPE	2-PGL	2-GML	2-EPL				2
309	2-PEM	2-LEPM	2-EML		2-EPL			2
320			2-E	3-EPL			2-EPL	2
141	2-EP	2-E	2-E				2-E	2
Total								26

Legend E = eosinophil; P = plasma cell; L = lymphocyte; M = macrophage; G = Globular leucocyte.
The order stated, indicates the most common cell first.
Score:- 1=mild, 2=moderate, 3=severe, 4=severe and widespread.

B, ANALYSIS of CELL PROPORTIONS

Total no. of slides examined = 40

No. where Eosinophils predominate = 29 (72.5%)		No. where Eosinophils seen = 37	92.5%
No. where Plasma cells predominate= 6 (15.0%)		No. where Plasma cells seen = 33	82.5%
No. where Lymphocytes predominate= 3 (7.5%)		No. where Lymphocytes seen =28	70.0%
No. where Globular Leucocytes pred = 2 (5.0%)		No. where Macrophages seen = 5	12.5%
		No. where Globular Leuc.seen = 3	7.5%

Appendix 4.7 Parasitology results in transmission experiment.

CALF	EGG COUNT	ABOMASAL WORM COUNT					SMALL INTESTINAL WORM COUNT			
Calf Number	Strongylate Eggs/gm	Haem.	Ost.	Trich.	Abo L4	Total Worm Count	N-dirus	Coop.	Sl L4	Total Worm Count
340	400									
301	50									
306	500	400	5600	18000	0	24000	200	4100	100	4400
332	600	200	3400	7300	0	10900				
324	150									
334	650	0	2800	3300	0	6100	100	2800	0	2900
335	800									
329	800	0	5600	11600	0	17200	0	7000	0	7000
309	0									
320	950	0	12900	8800	0	21700	0	4400	0	4400
141	-									
Sum	4900	600	30300	49000	0	79900	300	18300	100	18700
Mean	**490**	**120**	**6060**	**9800**	**0**	**15980**	**75**	**4575**	**25**	**4675**
Total										**20655 #**

LARVAL CULTURE	**6%**	**7%**	**87%**

Legend :- Haem. = Haemonchus species
Ost. = Ostertagia species
Trich. = Trichostrongylus species
Abo L4 = fourth stage larvae in abomasum.
N-dirus = Nematodirus species
Coop. = Cooperia species
Sl L4 = fourth stage larvae in small intestine.
= mean total worm burden