EPIDEMIOLOGY OF INFECTION WITH BOVINE VIRAL DIARRHOEA VIRUS IN A DAIRY HERD.

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Abstract

This thesis describes studies on epidemiological aspects of infection of a dairy herd with bovine viral diarrhoea virus. Serum neutralizing antibodies were measured and isolation of virus was attempted from animals in one commercial dairy farm and from bobby calves collected from dairy farms in the Manawatu Region for slaughter at a local abattoir.

The work is presented in six chapters. The first chapter is a review of the literature relevant to the present work. The second is a description of the general materials and methods. The third, fourth and fifth give the results of the investigations carried out and the sixth is summary, a general discussion and conclusion of the major findings. References are cited at the end of the thesis. Appendices I and II describe the preparation of the media for cell culture, reagents and solutions, and publications related to the work.

All of the replacement heifers and 97.1% of the adult milking herd had serum neutralizing antibodies to bovine viral diarrhoea virus. There was no significant difference ($p > 0.05$) in the geometric mean titres of each age group of the animals in the herd.

The serum antibody titres of the cohort of one to four day old calves reflected those of their dams and declined steadily, with a half life of 25 days, so that by eight months of age, all calves tested negative. The serum neutralizing antibody titres in a group of bobby calves at a local abattoir were significantly lower ($p < 0.001$) than those of the cohort of calves on the farm.

Bovine viral diarrhoea virus was isolated from blood samples from 21 of 340 (6.1%) animals and five (1.4%) of these animals were still infected when retested 12 months later. The distribution of serum neutralizing antibodies in infected animals ranged from < 0.6 to 2.7. Three of the persistently infected animals had no detectable neutralizing antibodies (titres < 0.6) but two had titres of 0.9 and 1.8. The age of the infected animals ranged from one to more than eight years.

Persistently infected animals provided an adequate mechanism for the maintenance of bovine viral diarrhoea virus in the herd. It is postulated that

animals, in contact with persistently infected animals, may be reinfected when their antibody titres decline below protective levels.

The viral interference assay and fluorescent antibody test were used to detect virus in cultures of blood samples. These two methods were evaluated for sensitivity and specificity and appeared to be equally suitable for virus isolation. In the present work there was 88.4% agreement between the two tests, while the viral interference assay was found to be relatively more sensitive but less specific than the fluorescent antibody test.

The results of this work indicate that infection with bovine viral diarrhoea virus is endemic on the farm studied and that most animals become infected by the time they are twelve months old. However, depending on the particular management practices used, some groups of heifer calves may not be exposed to virus before they reach breeding age. It also appears that infection with bovine viral diarrhoea virus is common on many dairy farms in the Manawatu Region. If identification and elimination of persistently infected animals is an option for the control of bovine viral diarrhoea virus in an endemically infected population, a laboratory test with high specificity and sensitivity and which is easy to perform will be required.

If eradication of BVDV from the farm is not practical then immunization of heifers around the age of 12 months, either by exposure to persistently infected animals in the herd or by using inactivated vaccines,.may protect them against infection during the first breeding period thus minimizing foetal infection.

STATEMENT

This is to certify the work on which this thesis is based has not been accepted in whole or in part for any other degree or diploma, and was carried out by the undersigned. Assistance received is specifically recorded in the Acknowledgements section bound with the thesis.

Abraham Gopilo Golo
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CHAPTER ONE

Review of the literature.

History

Bovine viral diarrhoea virus (BVDV) is a versatile Pestivirus which infects a wide range of wild and domestic ruminants and pigs and is an economically important pathogen of cattle throughout the world causing a wide range of clinical syndromes (Ames, 1986; Brownlie *et al.*, 1987).

The disease associated with infection with BVDV was first recognized in the United States of America (USA) in 1946 in association with epidemics of acute and often fatal disease which were characterized by diarrhoea and erosive lesions of the digestive tract (Olafson *et al.*, 1946). Since the disease was transmissible with material which contained no visible organisms it was named virus diarrhoea (Olafson *et al.*, 1946). Concurrently, similar cases with variations in the degree of severity, chronicity and prevalence were described and named mucosal disease (MD) (Ramsey and Chivers, 1953).

The virus of bovine viral diarrhoea (BVD) was discovered when agents cytopathic for bovine kidney cell cultures were recovered from animals with MD (Underdahl *et al.*, 1957). The aetiological connection between BVDV and the recognized disease syndromes was established when a cytopathic virus was recovered from a calf that died from viral diarrhoea (Gillespie *et al.*, 1960).

For some time it was thought that several different infectious diseases were associated with the condition now known as BVD-MD viral infection. Later however, viruses isolated from BVD and MD were shown to be serologically similar and, in experimental infections, caused the same mild diarrhoea (Gillespie *et al.*, 1961; Thomson and Savan, 1963).

Thomson and Savan (1963) performed clinical, pathological and virological comparisons of the syndromes and confirmed that BVD and MD were a single entity, caused by the same virus. They proposed the name virus

diarrhoea to embrace the two diseases. Later it was established that various strains of BVDV,whether inducing a cytopathic effect in cell culture or not, were antigenically related since all strains showed some degree of cross reactivity by fluorescence, neutralization, complement fixation and immunodiffusion precipitation tests (Castrucci *et al.*, 1975; Gillespie *et al.*, 1961; Fernelius *et al.*, 1971).

The naming of the virus is unfortunate even today, because of the multiple and diverse clinical manifestations which range from apparently healthy but persistently infected animals, to classical MD,and from subtle intrauterine growth retardation to overt congenital defects in the foetuses (Baker ,1987). The term mucosal disease complex was in common usage in the literature for a long time. In 1968 the *ad hoc* Committee on Terminology (Symposium on Immunity to the Bovine Respiratory Complex) recommended the name bovine viral diarrhoea-mucosal disease (BVD-MD) (Jensen *et al.*, 1968). The *ad hoc* Committee on Terminology for the American Veterinary Medical Association Symposium (1971) also supported the name bovine virus diarrhoea -mucosal disease (Timoney *et al* 1988). Many reports,however still continue to refer to the disease as bovine diarrhoea (BD), bovine viral diarrhoea (BVD), or mucosal disease (MD).

Throughout this thesis the virus is referred to as bovine viral diarrhoea virus (BVDV) and the infection as BVDV infection. The name mucosal disease (MD) will be used in limited circumstances to indicate the clinical features of BVDV infection, when persistently infected immunotolerant animals are superinfected with a cytopathic biotype of BVDV in postnatal life and develop the classical clinical syndrome associated with this infection.

This brief review of the literature consists of a description of the virus, with special reference to characteristics relevant to work in this thesis including the disease syndromes caused in cattle and other species as a result of infection, the epidemiology of infection of cattle with BVDV and control strategies.

The virus.

The genome of BVDV has been shown to be an infectious,positive single stranded RNA (Hafez and Liess, 1972; Renard *et al.*, 1985). The virion

consists of a core particle surrounded by a membranous envelope with numerous projecting knobs (Chu and Zee,1984). The subunits of the core are arranged in an icosahedral surface lattice or a cubic symmetry (Maess and Reczko, 1970). Structural polymorphism for BVDV particles has been observed (Chu and Zee, 1984; Gray and Nettleton, 1987; Ohmann and Bloch, 1982).

Together with the viruses of swine fever and border disease of sheep,the BVDV belongs to the genus Pestivirus in the family Togaviridae (Westaway *et al.*, 1985). Currently , however, molecular features of the viral replication suggest that it is more appropriately classified as a member of the family Flaviviridae (Collett *et al.*, 1988).

The virus is readily inactivated by lipid solvents such as ether, chloroform and sodium desoxycholate (Hermodson and Dinter, 1962; Hafez and Liess, 1972; Tanaka *et al.*, 1968). The growth of BVDV is also inhibited by proflavine or acriflavine (Brinton, 1980) and it is inactivated by trypsin (Hafez and Liess, 1972). The virus is susceptible to common disinfectants such as chlorhexidine, lysol,iodophors, aldehyde and hypochlorites (Duffel and Harkness, 1985).

The viability of BVDV under various physical conditions has been studied. The virus was rapidly inactivated at 56^{o}C in one hour (Taylor *et al.*, 1963). At 37^{o}C inactivation occurred more slowly with a total loss of viability in five days (Tanaka *et al.*, 1968). At room temperature (approximately 20^{o}C), no significant drop in viral titre in 24 hours has been observed (Coggins, 1964). Successful isolation of BVDV from blood after a delay of five days at room temperature (20^{o}C) between specimen collection and inoculation in cell culture has been reported (Rae *et al.*, 1987). At a temperature of four degrees Celsius or less BVDV appeared to be stable for at least 35 days (Tanaka *et al.*, 1968). The virus was found to be viable at -20^{o}C for six months in whole blood and lymph nodes (Ssentengo, 1978). This author also recovered infectious virus from material stored for 16 months at -40^{o}C. BVDV withstood up to ten cycles of repeated freezing and thawing (Parks *et al.*, 1972).

BVDV is stable over a pH range from 5.7 to 9.3 with maximum stability at pH 7.4 (Hafez and Liess, 1972). Stability within a pH range from three to nine has also been recorded (Duffel and Harkness,1985).

The virus is readily inactivated by ultraviolet irradiation. A 20 watt germicidal lamp, at a distance of 20 cm inactivated the virus completely after 140 seconds of exposure (Tanaka *et al.*, 1968). It was also inactivated after exposure to eight microwatts per cm^2 of UV irradiation for 90 minutes (Philip, 1973). The stability and viability of BVDV in the natural farm environment has not been determined in detail but it does not survive in the environment beyond 14 days (Duffel and Harkness, 1985).

Antigenic properties of BVDV have been determined. The virus does not haemagglutinate or haemadsorb red blood cells of birds or mammals (Castrucci *et al.*, 1968; Hafez and Liess, 1972). Viral antigens have been detected by serum neutralization (Gillespie *et al.*, 1961; Harkness *et al.*, 1978), immunodiffusion precipitation (Darbyshire, 1962), immunofluorescence (Fernelius, 1964), complement fixation (Gutekunst, 1964) and by enzyme linked immunosorbent assay (ELISA) (Bock *et al.*, 1988; Chu *et al.*, 1985; Howard *et al.*, 1985).

All strains of BVDV show some degree of cross reactivity (Bolin *et al.*, 1988; Castrucci *et al.*, 1975; Gillespie *et al.*, 1961; Peters *et al.*, 1986). Both cytopathic and noncytopathic viruses reacted with fluorescein conjugated serum globulins produced against either cytopathic or noncytopathic viruses, but the fluorescence was more intense in the homologous system (Fernelius, 1964). Monoclonal antibody analyses have also demonstrated antigenic relationships and differences, and a number of strains of both the cytopathic and noncytopathic biotypes are now recognized (Bolin *et al.*, 1988; Peters *et al.*, 1986).

Propagation of BVDV in tissue culture was first described in 1957 (Underdahl *et al.*, 1957). It was initially propagated in primary bovine kidney and testis cell cultures (Gillespie *et al.*, 1960; Gutekunst and Malmquist, 1963). Isolates differ in their cytopathic effects in cell culture and are commonly grouped as cytopathic or noncytopathic biotypes. The cytopathic strains cause an obvious cellular degeneration in a range of bovine cells grown *in vitro*. Some strains maintain their cytopathogenicity in a wide range of tissues while the cytopathogenicity of others, changes during passage in the same or different cell cultures (Fernelius and Lambert, 1969).

Sensitivity of a variety of cell monolayers to BVDV has been compared (Hoezinek, 1981; Malmquist, 1968). The most extensively used cell cultures for propagation and isolation of BVDV have been bovine foetal kidney cells, bovine foetal testicular cells, bovine embryonic spleen cells, bovine foetal lung cells, bovine foetal tracheal cells, bovine embryo skin-muscle cell lines, hamster kidney (HaK) cell lines and human cells resembling HeLa cell lines (Fernelius and Lambert, 1969; Goldsmit and Barzilai, 1975; Horzinek, 1981; Straver *et al.*, 1983).

Identification of noncytopathic strains became possible when a plaque inhibition assay was employed to detect noncytopathic strains of BVDV (Gillespie *et al.*, 1962). The assay was based on the principle of viral interference. Interference between strains of BVDV has been reported (Gillespie *et al.*, 1962). Cultures became resistant to challenge with a cytopathic strain three days after infection with a noncytopathic strain (McKercher *et al.*, 1968). Interference has also been reported between BVDV and bovine entroviruses (Schiff and Storz, 1972). BVDV is also sensitive to inhibition by interferon (Brinton, 1980). An immunofluorescence test has also been used for detecting and titrating noncytopathic viruses (Fernelius, 1964).

The presence of inclusion bodies in cells infected with BVDV *in vivo* and *in vitro* has been reported, but their specificity must be questioned (Horzinek, 1981). Large intracytoplasmic accumulations of ribosome-like particles have also been recorded at six to 12 hours post infection (Ritchie and Fernelius, 1969).

The disease

Infection of susceptible animals is by the oropharyngeal route with primary replication occurring in the tonsil and retropharyngeal lymph nodes. Subsequent viraemia spreads the virus to lymphoid tissue throughout the body particularly mesenteric lymph nodes, spleen and Peyer's patches (Ohmann, 1982). Viral antigen has been detected in the thymus, intestinal crypts and villi, lymph nodes, Peyer's patches, bronchi, the basal layer of the skin, oesophagus and spleen (Kendrick, 1971, Ohmann, 1982).

Macrophages are involved in the uptake of BVDV and its introduction from the portal of entry into the vascular and lymphatic systems (Truit and

Shechmeister, 1973). A high-titre, cell associated viraemia spreads the virus to various parts of the body resulting in an extensive distribution. BVDV also replicates in mononuclear phagocytes (Ohmann, 1983). Other studies also revealed that BVDV infects and replicates in subpopulations of all the four major mononuclear cell groups in the peripheral blood; i.e., T and B lymphocytes, monocytes and null cells; of these the B cell population makes only a minor contribution (Ohmann *et al.*, 1987). Leucopaenia which occurs during infection of cattle with noncytopathic strains of BVDV is characterized by a significant decrease in the absolute number of both major subsets of T and B lymphocytes and neutrophils (Ellis, 1988). Cattle experimentally infected with cytopathic strains of BVDV also have a transient decrease in both the percentage and absolute number of circulating T cells (Bolin *et al.*, 1985).

Sequelae of infection depend on age, and pregnancy status of the animal and whether it is a primary postnatal infection with a noncytopathic strain or superinfection with a cytopathic strain of BVDV (Ames, 1986; Grotelueschen and Mortimer, 1988; Perdrizet *et al.*, 1987). The disease syndromes caused by BVDV include BVD, MD, foetal disease and reproductive disorders (Brownlie, 1985; Grotelueschen and Mortimer, 1988; Perdizet *et al.*, 1987).

Bovine viral diarrhoea (BVD).

The majority (70-90%) of infections in suceptible adult cattle are subclinical or a mild transient disease may occur (Ames, 1986; Brownlie *et al.*, 1987; Shimizu *et al.*, 1989). However, the infection whether subclinical or not, will render the animals transiently immunosuppressed (Edwards *et al.*, 1986; Nagele, 1984; Potgieter, 1988).

The clinical consequence of BVDV induced immunosuppression depends on several circumstances, such as environmental or managemental stress and concurrent infection (Potgieter, 1988). It has been suggested that BVDV may have a major role in the aetiology of neonatal calf diarrhoea (Lambert *et al.*, 1974) and also may be an important respiratory tract pathogen (Potgieter, *et al.*, 1984, Potgieter, 1988)

An epidemic of BVDV infection in a susceptible closed herd may result in clinical signs in up to 25% of cattle and follow an incubation period of five to seven days (Blood *et al.*, 1983). Signs include nasal and/or ocular

discharge, fever, oral erosions or ulcerations, anorexia, mild depression and diarrhoea. Morbidity may be high but the mortality rate is low (Baker, 1987). Affected animals generally develop diarrhoea two to four days following the onset of the disease and neutrophilia, leucopaenia and viraemia persist for up to 15 days (Brownlie, 1985; Duffel and Harkness, 1985). Animals with acute BVD have leucopaenia, are immunosuppressed and may be more susceptible to secondary or concurrent bacterial or viral disease (Brownlie, 1985). Such mixed infections have been reported from both field and experimental work and may represent an important sequel of acute BVD (Malmquist, 1968; Reggiardo, 1979; Potgieter *et al.*, 1984; Potgieter, 1988). Synergism of BVDV with *Pasteurella haemolytica* and the viruses of parainfluenza, infectious bovine rhinotracheitis and respiratory syncytial virus have been observed in respiratory syndromes associated with these agents (Potgieter *et al.*, 1985).

Primary post-natal infection occurs most commonly in cattle aged from six months to two years (Blood *et al.*, 1983). Up to six months most of the calves are protected by maternal antibody. However, the duration of maternally derived antibody to BVDV in calves varies considerably and depends on the titre of the antibody in the dam's serum and the colostrum, and also the amount of colostrum ingested (Kahrs, 1981; Stober, 1984). Maternally derived antibodies usually decline to undetectable levels by six to 12 months of age (Coria and McClurkin, 1978, House and Manley, 1973; Kahrs *et al.*, 1966; Kendrick and Franti, 1974; Stober, 1984).

Recovered animals develop neutralizing antibody three to four weeks following infection. Once the neutralizing antibody reaches a peak, which usually occurs ten weeks post infection, it remains high and is associated with immunity which lasts for life (Baker, 1987; Brownlie *et al.*, 1987; Kahrs, 1966). Cattle with lower antibody titres however, may show evidence of subsequent reinfection by further serological responses (Brownlie *et al.*, 1987; Coria and McClurkin, 1978). Low antibody titres can undoubtedly be overcome by massive challenge with BVDV and by some live vaccines (Brar *et al.*, 1978).

The serum neutralization (SN) test is a most useful tool for serological investigation of the herd for BVDV infection. However it is of limited use for initial diagnosis because affected animals may be negative in the first few days of acute infection (Littlejohns, 1988).

Mucosal disease (MD).

MD occurs sporadically in cattle, usually between the age of six months and two years. Less than five per cent of the herd is affected, but the case fatality rate approaches 100% (Duffel and Harkness, 1985).

Since many attempts to experimentally reproduce MD in normal cattle have failed, the pathogenesis of the disease has remained obscure for a long time. The results of clinical observations and detailed virological investigations have revealed a consistent association of cytopathic virus with MD (Barber *et al.*, 1985; Brownlie *et al.*, 1984).

Malmquist (1968) suggested that MD is a late sequel of an immunotolerance established by *in utero* infection. This hypothesis has been supported by more recent investigations (Nagele, 1984; Ohmann, 1988; Roeder and Drew, 1984; Steck *et al.*, 1980). Recently the disease has been reproduced experimentally in persistently infected cattle following inoculation with cytopathic virus (Bolin *et al.*, 1985; Brownlie *et al.*, 1984). However the precise mechanisms involved in the pathogenesis of MD remain poorly understood (Littlejohns, 1988; Shimizu *et al.*, 1989).

Both cytopathic and noncytopathic viruses have been isolated from individual cases of naturally occurring MD (Bolin *et al.*, 1985; Howard *et al.*, 1987; McClurkin *et al.*, 1985) and the two biotypes of BVDV isolated from MD have been found to be closely related antigenically (Howard *et al.*, 1987). Brownlie and others reported that all persistently infected cattle succumbed to MD within two to three weeks of being challenged with a closely related cytopathic strain of virus. BVDV isolates other than from MD, are almost always noncytopathic (Barber *et al.*, 1985; Brownlie, 1985). It has been postulated that cytopathic virus continually arises, possibly by mutation from the noncytopathic virus. This may account for the antigenic similarity of noncytopathic and cytopathic isolates from naturally occuring MD (Brownlie *et al.*, 1987; Howard *et al.*, 1987).

The clinical signs of acute and chronic MD have been extensively described (Ames, 1986; Brownlie, 1985; Duffel and Harkness, 1985; Grotelueschen and Mortimer, 1988). The clinical signs of MD develop approximately seven to ten days after exposure to cytopathic BVDV and progress until death supervenes. Sudden death may,in fact, be the first clinical sign observed. Affected animals

typically succumb in three to ten days but may survive for as long as three weeks. The most prominent clinical sign is the appearance of profuse, watery, foetid diarrhoea, which may contain fresh or clotted blood. As the disease progresses, dehydration leads to severe weight loss, depression and death. Excessive salivation commonly occurs as a result of ulceration of the gums, tongue and buccal mucosa. Erosions, also seen in the nasal cavity and on the muzzle, lead to crusting and a mucopurulent discharge. Erosions may also be present in the conjunctiva, interdigital clefts, and the mucocutaneous junction of the vulva or the prepuce. Affected animals may be reluctant to move due to laminitis and coronitis. In lactating cattle milk production decreases dramatically.

A small propotion of cattle that have MD do not die within the expected time frame and become chronically affected. The characteristic diarrhoea may be intermittent. These animals often present with chronic bloat, weight loss, nonhealing skin lesions, alopecia, hyperkeratinization, interdigital lesions, gastrointestinal erosions, mucocutaneous erosions and severe emaciation (Stober, 1984). Cattle with chronic MD may survive up to 18 months and ultimately die from severe debilitation. They should be distinguished from those calves born persistently infected with noncytopathic BVDV, which are unthrifty and " poor doers " from birth. Such calves may develop MD but also may succumb to other infections, in which case signs and lesions of MD would not be seen.

Pathological lesions are most obvious in the gastrointestinal tract with small erosions, larger areas of necrosis and sloughed epithelium throughout the tract (Brownlie,1985). Lesions in other parts of the body include bronchopneumonia, similar to that normally associated with bacterial infection. Emphysema of the lungs, haemorrhages in the epicardium and on the serosal surface of the ileum have also been observed (Ruth, 1986). Externally, coronitis and interdigital lesions, characterized by epithelial necrosis with marked inflammation of the underlying dermis are commonly seen. In chronic cases, the skin will be dry and rough. Decubital ulcers and arthritis of the carpal and knee joints occur in chronically debilitated animals (Binkhorst *et al.*, 1983).

MD is only one of the clinical manifestations of disease produced as a result of infection of cattle with BVDV. Therefore a diagnosis of MD can justify

the need for a thorough investigation of virological and epidemiological facets of the disease in the rest of the herd. Usually the clinical history, signs and virus isolation (from blood and/or tissues) are useful criteria in the diagnosis of MD. However, the results of serological tests require careful interpretation, because animals that develop MD are always antibody negative (Baker, 1987).

Infection of the foetus.

Clinical evidence accumulated from studies of natural outbreaks indicates that abortions are a consistent finding (Dow *et al.*, 1956; Olafson *et al.*, 1946). Foetal infection at about 50 to 100 days of gestation may result in foetal death follwed by abortion or mummification (Done *et al.*, 1980). Abortion and early embryonic death with resorption are observed clinically as a repeat breeder problem (Roeder and Drew, 1984; Roeder and Harkness, 1986). However, expulsion of the foetus may occur up to several months after infection (Kahrs, 1981). In more general terms BVDV appears to cause only a small proportion of all abortions (Stober, 1984).

Persistent infection and immunotolerance to BVDV occurs following infection of the foetus before complete maturation of the foetal immune system. This occurs with infection between 42 days and 125 days of gestation (Coria and McClurkin, 1978; Cutlip *et al.*, 1980; McClurkin *et al.*, 1984). Persistent infection following foetal infection appears to be associated exclusively with infections with noncytopathic biotypes of BVDV (McClurkin *et al.*, 1985). Cattle immunotolerant to BVDV are persistently infected, viraemic and constantly shed virus into the environment (Barber *et al.*, 1985; Brownlie *et al.*, 1987).

Immunotolerance to BVDV has been confirmed by a lack of specific antibody in either the developing foetus or in animals after birth. The immune system is not fully functional before 125 days of gestation and infection with BVDV up to this age results in the virus becoming widely established in the foetal tissues. Subsequently, although the immune system matures, it recognizes the virus as self and a state of specific immunotolerance to the infecting noncytopathic virus develops. It is this tolerance, that allows the the virus to persist into postnatal life. However, there is no evidence so far, from either field or experimental work, that a persistent viraemia occurs with cytopathic strains of BVDV (Brownlie *et al.*, 1987; Coria and McClurkin, 1978). Immunotolerance appears to be specific to the infecting noncytopathic strain of

virus, because persistently infected cattle are capable of recognizing certain epitopes of heterogenous strains of BVDV and producing specific antibody (Howard *et al.*, 1987; Ruth, 1986).

Some of the persistently infected animals are stunted and unthrifty (Barber *et al.*, 1985; Kendrick,1971), however, a proportion of the persistently infected animals remain apparently healthy for several years and live to maturity or breeding age and can represent 0.5-2% of the herd (Cutlip *et al.*, 1980; Ernst *et al.*, 1983; Howard *et al.*, 1986; Kahrs,1981; Meyling, 1984; Stober, 1984). Potgieter, (1988), summarized the postnatal sequelae of persistently infected immunotolerant animals :

- they may die prematurely from secondary infections or environmental stress;
- they have a high probability of developing MD;
- they constitute an important source of BVDV to the herd.

Diagnosis of persistently infected animals has an epidemiological significance. These animals usually do not have SN antibodies or rarely may have low titres. False positive reactions because of interference from high titres of noncytopathic virus in the serum should be taken into consideration (Baker, 1987). Virus isolation is important if control measures are to be considered.

Foetal infections between 100 and 150 days of gestation can result in a variety of congenital defects (Binkhorst *et al.*, 1983). Cerebellar hypoplasia is the most commonly observed malformation. Occasionally hydrocephalus, hydranencephaly with or without cranial deformation, defective myelination of the spinal cord (Badman *et al.*, 1981; Done *et al.*, 1980; Markson *et al.*, 1976) and ocular defects such as lens opacity, retinal atrophy or degeneration, optic neuritis and microphthalmia may be detected (Binkhorst *et al.*, 1983; Bistner *et al.*, 1970; Brown *et al.*, 1975; Ward *et al.*, 1969).

The corresponding neurological signs in the liveborn calves are incoordination or total inability to stand despite a normal muscle tonus and sucking reflex (Done *et al.*, 1980; Markson *et al.*, 1976; Scott *et al.*, 1973). Blindness, nystagmus, opisthotonus or tilted head have also been obseved (Kahrs *et al.*, 1973; Markson *et al.*, 1976; Scott *et al.*, 1973). Growth retardation as well as thymic hypoplasia have been described after BVDV was

inoculated into foetuses between 100 and 150 days of gestation (Brown *et al.*, 1974; Done *et al.*, 1980).

Infection of foetuses with BVDV in the later stages of gestation after the development of immunocompetence, rarely causes congenital malformations. Such calves may be normal at birth and have neutralizing antibodies. These calves are not persistently infected and are resistant to later challenge (Casaro *et al.*, 1971; Muscoplat *et al.*, 1973). The presence of SN antibodies in serum which has been collected before the ingestion of colostrum indicates that the calves were infected *in utero* during the last trimester.

Reproductive effects

BVDV can cause various reproductive dysfunctions depending upon the time of exposure to the virus (Grotelueschen and Mortimer, 1988). BVDV has been associated with ovaritis in infertile heifers (Ssentongo *et al.*, 1980). Seronegative females, inseminated with BVDV contaminated semen, have required increased numbers of services per conception. Seroconversion to BVDV is a possible requirement for pregnancy in these animals (McClurkin *et al.*,1979). Decreased semen quality has been reported in persistently infected bulls (Schultz *et al.*, 1982; Barlow *et al.*, 1986; Coria and McClurkin, 1978).

Interference with fertilization has been reported when BVDV is present in the uterus of seronegative females near the time of insemination (Grahn *et al.*, 1984). Ova collected three days post insemination from superovulated cows showed a 52% fertilization rate for virus treated animals, compared to 81.6% for control animals. Nearly identical percentages were obtained when embryos were collected on day 13 in another phase of the experiment (Grotelueschen and Mortimer, 1988).

Infection of other animals with pestivirus.

Although BVDV most commonly infects cattle, natural infection also occurs in sheep, goats,pigs, deer and wild ruminants (Baker, 1987; Doyle and Heuschele, 1983; Nettleton and Herring, 1980). The virus has also been isolated from a batch of equine serum (Horner, personnal communication, 1988). However natural infection of horses is not known to occur. Presumably this virus originated from contamination with infected bovine serum during processing.

Border disease.

Border disease (BD) of sheep also known as "Hairy Shaker" disease (HSD), was first described in Britain in flocks of the Welsh border counties (Hughes *et al.*, 1959). The disease is characterized in lambs by hairy birth coats, poor growth and viability and central nervous system disturbances with tremor (Hartley and Kater, 1962). Similar clinical syndromes have been described in New Zealand (Manktelow *et al.*, 1969), Ireland (Hamilton and Donnelly, 1970), the United States (Osburn *et al.*, 1972) and Australia (Barlow and Dickinson, 1965).

Transmission studies on BD were carried out by injecting a crude suspension of brain, spinal cord and spleen from affected lambs into ewes in early pregnancy. These studies established the infectious nature of BD (Dickinson and Barlow, 1967; Gardiner and Barlow, 1972; Manktelow *et al.*, 1969; Shaw *et al.*, 1967).

Currently, border disease virus (BDV) is classified as a Pestivirus which is antigenically related to BVDV and swine fever virus (SFV) (Plant *et al.*, 1973). Although BDV is antigenically related to BVDV and SFV, it is also possible that BD is caused by some strains of BVDV that are present in both cattle and sheep populations (Barlow and Patterson, 1982; Horzinek, 1981; Nettleton *et al.*, 1986). An association between BVDV and BDV and the clinical syndromes in sheep has been demonstrated experimentally (Plant *et al.*, 1976) and affected lambs with BD can be sources of pestivirus infection for cattle (Barlow *et al.*, 1980). Sheep infected with BD had neutralizing antibodies to BVDV and BDV (Barlow *et al.*, 1979). In addition BVDV and BDV may cross infect between cattle and sheep and BVDV readily crosses the placenta of either species and induces comparable congenital defects (Gibbons *et al.*, 1974); Terlecki *et al.*, 1980).

Congenital defects have been observed in lambs, whose dams were experimentally infected with BDV at 54 days of gestation (Barlow , 1980). Cerebellar hypoplasia and dysmyelination of the central nervous system is a characteristic feature of BD and may lead to congenital tremor in the newborn lambs (Barlow, 1980).

Infection of ewes between 21 and 72 days of gestation with BVDV resulted in the birth of persistently infected lambs (Terpstra, 1981). After

approximately 80 days of gestation all foetuses which are exposed to BDV mount an effective immune response, eliminate infection and their presucking sera contains SN antibody (Roeder *et al.*, 1987). A pregnant ewe which is infected during pregnancy develops a high antibody titre, clears the virus and is no longer a source of BDV (Sawyer *et al.*, 1986). BDV has been isolated from secretions and excretions of persistently infected lambs for many months after birth (Potts *et al.*, 1985; Sawyer *et al.*, 1983). Experimental studies demonstrated that infected lambs, at one month of age shed virus in sufficient titre to infect ewes which were in contact with them (Sawyer *et al.*, 1986).

Infection of goats with BDV or BVDV probably induces a similar pattern of disease, but there is very limited information in the literature (Baker, 1987). Clinical and virological studies in other ruminants have attracted investigators only after clinical signs similar to MD have been observed.

Infection of pigs.

Antigenic relationships between BVDV and SFV have been demonstrated by agar gel diffusion precipitation and immunofluorescence (Darbyshire, 1962; Mengeling *et al.*, 1963). Pigs which have been exposed to certain strains of BVDV or SFV can develop neutralizing antibody to both BVDV and SFV (Corthier *et al.*, 1974). In a group of pigs which were exposed to a field strain of BVDV, moderate to high antibody titres against BVDV and low cross neutralizing titres against SFV were detected (Stewart *et al.*, 1971).

Antigenic differences between BVDV and SFV in cross neutralization studies were also observed. Some strains of BVDV were more closely related to SFV than were others (Castrucci *et al.*, 1975). Monoclonal antibodies produced against SFV and used in immunoperoxidase tests with strains of BVDV did not recognize a number of strains of BVDV (Wensvoort *et al.*, 1986). Extensive cross reactions were detected when five monoclonal antibodies against the NADL strain of BVDV were tested with 12 heterologous strains of BVDV and four strains of SFV by indirect immunofluorescence assay. One antibody preparation reacted with all strains, two antibodies were specific for cytopathic strains of BVDV, but failed to react with SFV. The other antibodies reacted to varying degrees with both strains of BVDV and SFV (Peters *et al.*, 1986).

Natural and experimental infection of pigs with BVDV has been reported. Results of surveys have revealed that the prevalence of naturally

occurring antibodies to BVDV in pigs ranged from three to 40 per cent (Snowdon and French, 1976) and 15 % of sows slaughtered in the Netherlands had antibodies against BVDV (Terpstra and Wensvoort, 1988). Transplacental transmission of BVDV in sows without signs of disease in the piglets has been observed (Stewart *et al.*, 1980). Pigs which were exposed to BVDV during intrauterine life may become persistently infected and responsible for further dissemination of BVDV among pigs (Terpstra and Wensvoort, 1988). BVDV is shed in secretions and excretions of infected pigs for three weeks (Fernelius *et al.*, 1973). Most of the BVDV isolates recovered from pigs have been of the noncytopathic strain (Fernelius *et al.*, 1973). Cattle are regarded as the source of infection of pigs with BVDV. Feeding pigs with unprocessed bovine offal and vaccination with vaccines contaminated with BVDV are also sources of infection. Pigs in close contact with cattle have often become infected with BVDV (Stewart *et al.*, 1980; Wensvoort and Terpstra, 1988).

Experimental and natural postnatal infections of pigs with BVDV are usually subclinical except for an occasional mild pyrexia (Fenelius *et al.*, 1973). Other reports have described clinical signs and postmortem lesions that closely resembled chronic swine fever (Mengeling and Cheville, 1968).

Epidemiology of infection of cattle with BVDV.

Transmission.

The main sources of BVDV in the herd are either the acutely infected animals or those which are persistently infected and shed the virus into the surrounding environment (Bolin *et al.*, 1985; Duffel and Harkness, 1985; Heuschele, 1978; McClurkin *et al.*, 1985).

Transmission may be direct or indirect. Inhalation or ingestion of infected saliva, oculonasal discharge, urine and faeces are principal means of infection (Brownlie *et al.*, 1987; Duffel and Harkness, 1985). The major period of excretion following acute infection is from about day four to day ten, but the virus may be recovered from nasal swabs up to about day 19 after infection (Brownlie *et al.*, 1987). Faeces, however, are poor sources of virus even when there is severe damage to the gut (Brownlie *et al.*, 1987). BVDV does not persist in the environment beyond 14 days (Duffel and Harkness, 1985).

Close contact of susceptible animals is necessary for efficient direct transmission. The attack rate of BVDV, which has been computed to be in the range between 0.002 to 0.06 per susceptible animal per day in grazing cattle, increases to 60% when susceptible animals are yarded overnight with a persistently infected animal which is shedding virus (Littlejohns, 1988).

BVDV has been isolated from sheep, goats,pigs and wild ruminants and these species may serve as reservoirs (Baker, 1987). Border disease virus and BVDV have been shown experimentally to cross infect between cattle and sheep, and such transmission may also occur under natural conditions where these animals are farmed together (Duffel and Harkness, 1985; Nettleton, 1987).

Indirect transmission may be through vehicles such as contaminated feed and equipment or human beings, who have contact with infected animals. There is also the potential for transmission via contaminated hypodermic needles (Kahrs, 1981; Ohmann, 1983; Roeder and Harkness, 1986).

Transmission also may occur through infected semen, uterine secretions, amnionic fluid, or placentae (Ssentengo *et al.*, 1980; Stober, 1984). BVDV has been recovered from bovine semen from both persistently infected, immunotolerant bulls and acutely infected bulls (Barlow *et al.*, 1986; Coria and McClurkin, 1978; Meyling, 1988; Roeder and Drew, 1984; Schultz *et al.*, 1982; Whitmore *et al.*, 1977). However, the epidemiological significance of semen-borne transmission of BVDV has not yet been determined.

Transplacental infection of the foetus is well recognized (Done *et al.*, 1980; Harkness, 1987; Kendrick, 1971; Malmquist, 1968; Ohmann, 1982).

Various experimental transmissions of BVDV indicate that oral, nasal, intratracheal, intravenous, intramuscular and intrauterine administration of virus suspension results in infection (Lopez *et al.*, 1982; Mills and Luginbuhl, 1968; Ohmann, 1983; Potgieter *et al.*, 1984). The disease has been artificially transmitted to calves following intraperitoneal (IP) injection of a suspension of spleen which was obtained from a naturally infected animal which had been ill for 2 months (Dow *et al.*, 1956). Thomson and Savan (1963) succeeded in transmitting infection to calves by inoculating suspensions of various organs taken at postmortem from cases of mucosal disease. Experimental infections

with BVDV indicated that the virus crossed the placenta and infected the foetus with remarkable efficiency and foetal infection occurs in about half of the pregnancies at risk (Casaro *et al.*, 1971; Kahrs, 1973; Harkness, 1987).

Distribution of SN antibodies in cattle populations.

BVDV has a worldwide distribution. Serological studies provide evidence of BVDV infection with serum antibody prevalences in cattle ranging from nine up to 100% (Kahrs, 1981). The distribution of BVDV has been investigated in a number of countries. Serological investigations in North America indicated 50% to 90% of clinically normal cattle had neutralizing antibody (Ernst *et al.*, 1983). In Britain the disease was first described on 35 farms in 1956 (Dow *et al.*, 1956). Since then Darbyshire (1962) examined 757 sera using an immunodiffusion test and found that 30.6% of samples gave positive reactions. Over 4000 sera from dairy cattle were tested by the serum neutralization test and 61% were found to be positive (Philip, 1973). In other surveys of cattle in England and Wales, 1593 sera were examined by the serum neutralization test, and 62.2% showed a positive reaction (Harkness *et al.*, 1978).

The prevalence of neutralizing antibody in cattle surveyed in Africa ranged from 11.6 - 79.4%; in Europe from 9 - 100%; and in the Middle East 50% (Abraham and Barzilai, 1972). In Australia, 60% of the surveyed cattle were found to have neutralizing antibody (French and Snowdon, 1964). Serological surveys of cattle in New Zealand found neutralizing antibody to BVDV in 41-66% of those sampled (Fastier and Hansen, 1966; Robinson, 1971).

The prevalence of neutralizing antibody in the cattle population reflects the proportion of animals that have been exposed to the virus at some time during their life. The discrepancy between prevalence of antibody and the incidence of clinical disease indicates that most infections are subclinical or inapparent (Harkness *et al.*, 1978). However 1 - 3% of cattle in the herd may not have neutralizing antibody and yet be persistently infected and viraemic. They are constantly shedding BVDV into the environment and act as the major reservoir of the virus in the herd even though they may appear clinically healthy (Barber *et al.*, 1985; Bolin *et al.*, 1985; Cutlip *et al.*, 1980; Ernst *et al.*, 1983; McClurkin *et al.*, 1985; Meyling, 1984; Nettleton *et al.*, 1986).

The role of persistently infected immunotolerant animals.

When BVDV is introduced into a herd the natural spread of the virus appears to be by direct contact between animals. The majority of the infections in susceptible adult cattle are subclinical (Brownlie, 1985). The incubation period of five to seven days is followed by transient fever, leucopaenia and viraemia for up to 15 days, and virus is present in nasal and lacrimal secretions and in urine and faeces (Duffel and Harkness, 1985). Recovery is accompanied by the development of neutralizing antibody, which is generally detected in serum at two to four weeks after infection and may provide lifetime protection (Brownlie *et al.*, 1987; Kahrs *et al.*, 1966).

As a result of primary exposure of cattle to BVDV within the confines of a farm all groups of animals become infected. Thus, initial spread of the virus may be followed by immunity and subsequent elimination of BVDV from the farm. When the virus has been eliminated, and if there is no new introduction of infected cattle, a herd can be become virus free and, within a generation, antibody negative. Unfortunately these antibody negative, closed herds are highly susceptible to future infections with BVDV. It is not uncommon for the infection to be introduced by a recently purchased heifer or a new bull, with either a transient or persistent viraemia. The chance of spread of BVDV is much greater when the introduced animal is persistently infected. The presence of such an animal maintains a continued source of BVDV within the herd as opposed to a transiently infected animal which will only shed the virus from ten to 15 days. Being efficient transmitters of BVDV, persistently infected animals appear to be a major mechanism by which the virus is maintained in the cattle population (Brownlie *et al.*, 1987; Roeder and Harkness, 1986).

In some closed farms, replacement heifers are raised separately from the adult stock. With the waning of maternally derived immunity they become antibody negative and highly susceptible to infection with BVDV. If there is a persistently infected heifer in the group, the remainder of the animals may become transiently infected and then develop resistance to subsequent infection. If exposure to BVDV does not occur until after the heifers have become pregnant then damage to the developing foetus may occur depending on the stage of gestation at which infection occurred (Roeder and Drew, 1984).

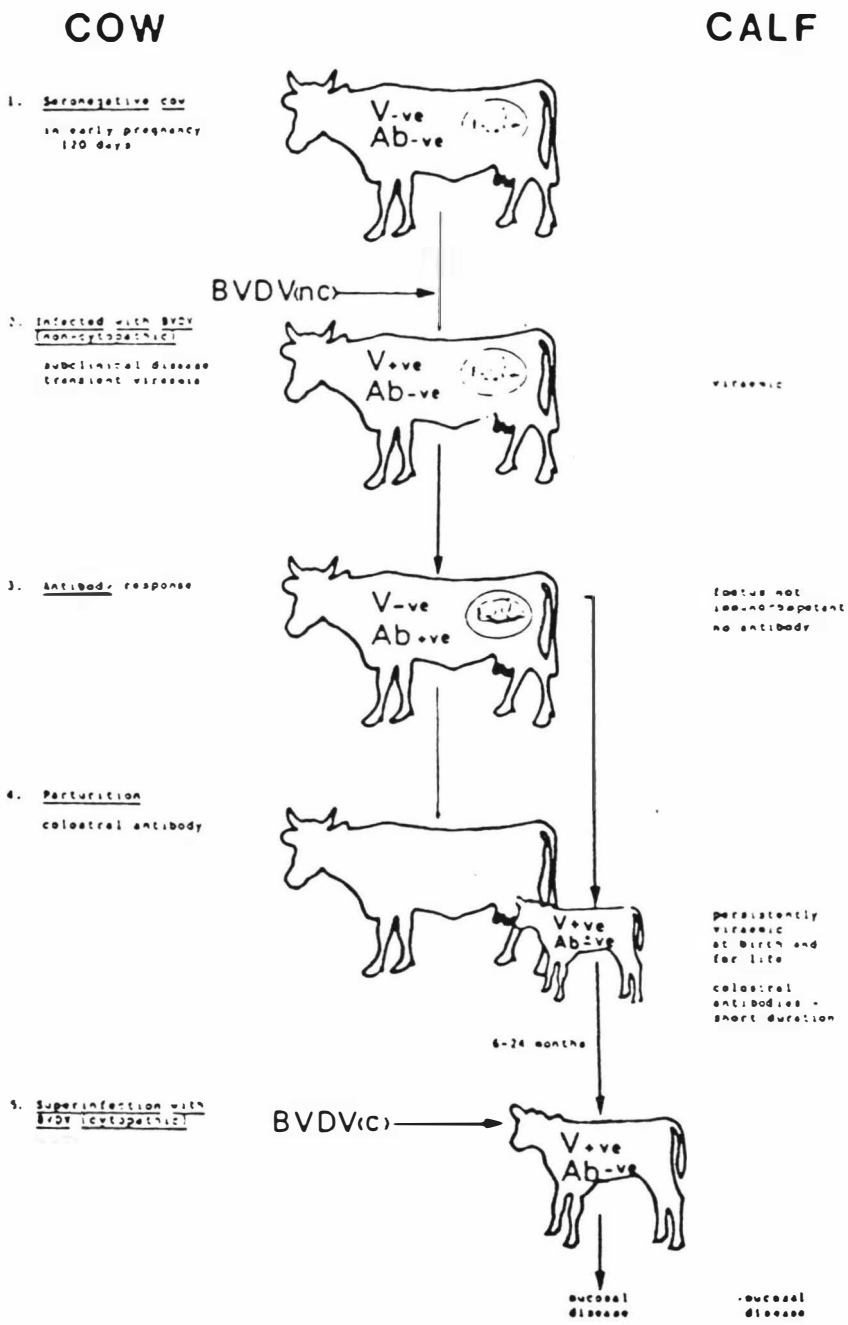
If persistently infected cattle reach breeding age, their offspring may be persistently infected and families of persistently infected cattle may develop

within a herd (McClurkin *et al.*, 1979; Straver *et al.*, 1983). Thus BVDV may be spread within the herd from generation to generation through persistently infected animals.

The presence of these persistently infected, immunotolerant, virus shedding animals and the continous presence of susceptible animals are necessary for the perpetuation of infection in the herd (Littlejohns, 1988). The significance of these immunotolerant, persistently infected animals in the epidemiology of infection of cattle with BVDV has been only relatively recently recognized (Brownlie *et al.*, 1987; Duffel and Harkness, 1985).

The epidemiological role of cytopathic BVDV is closely related to the pathogenesis of MD (Fig. 1-1). There is still debate however, over from just where the cytopathic strain originates from in naturally occurring MD i.e. exogenous infection or endogenous by mutation from the non-cytopathic strain (Brownlie *et al.*, 1987; Littlejohns, 1988).

Fig. 1-1. **Pathogenesis of Mucosal disease (Brownlie *et al.*, 1987).**


COW
CALF
1. Seronegative cow
in early pregnancy
120 days
V-ve
Ab-ve
BVDV(nc)
2. Infected with BVDV (non-cytopathic)
subclinical disease
transient viraemia
V+ve
Ab-ve
viraemic
3. Antibody response
V-ve
Ab+ve
foetus not immunocompetent
no antibody
4. Parturition
colostral antibody
V+ve
Ab+ve
persistently viraemic at birth and for life
colostral antibodies - short duration
6-24 months
5. Superinfection with BVDV (cytopathic)
BVDV(c)
V+ve
Ab-ve
mucosal disease
mucosal disease

Control of infection with BVDV.

General concepts of control.

Economic losses associated with infection of animals with BVDV include abortion, neonatal death and subsequent MD in persistently infected progeny (Duffel *et al.*, 1986). As epidemiological factors vary from herd to herd, and national statistics on annual incidence are not known, no accurate estimate of the cost of the disease to the cattle industry in New Zealand can be made.

It is feasible to control established BVDV infection within a herd, but not to prevent its introduction or spread (Roeder and Harkness, 1986). It may not be practical to maintain BVDV free herds because of the multiple routes by which the virus may enter i.e., contact with other ruminants, persistently infected replacement cattle, contaminated semen, embryo transfer (there may be a potential for BVDV contamination of bovine foetal serum used in this procedure), contaminated hypodermic needles, use of modified live virus vaccines in pregnant animals, and contamination of biological products with adventitious BVDV (Baker, 1987). Therefore, with the virus being so common, there is always a risk of reinfection of a herd from which infection had been previously eliminated, with the possibility of a much higher and unacceptable level of endemic infection (Littlejohns, 1988).

Programmes for prevention of infection must be designed to fit the individual type of farm operation (dairy, calf raising, feedlot, etc) as well as be compatible with the rest of the farm's animal health and production programme (Ernst *et al.*, 1983). The current concept of the epidemiology of BVDV infection indicates clearly that, in breeding herds, the primary aim of control should be the prevention of prenatal infection (Harkness, 1987). Prevention of transplacental infection may be achieved by ensuring that all breeding animals in the herd are immune before they are mated (Baker, 1987). Controlled exposure of susceptible cattle to persistently infected cattle to induce immunity has also been suggested (Littlejohns, 1988). Although the prevention of transplacental infection is an extremely critical area of focus for control, it is still important to consider the role of the virus in causing immunosupression, particularly in stressed cattle. Therefore it would be advantageous to have established immunity to BVDV in calves before their arrival at a feedlot (Ernst *et al.*, 1983).

The possible options.

i) Deliberate inactivity. This is a non-intervention option, which is tenable only in breeding herds when BVDV infection has spread rapidly through the herd stimulating high levels of immunity. This policy has unpredictable consequences and provides only short term solutions in the absence of additional measures (Harkness, 1987).

ii) Removal of persistently infected animals. Identification and removal of persistently infected animals followed by stringent precautions to ensure long term freedom from infection (Harkness, 1987; Brownlie, 1985).

Advantages of this approach include elimination of a continual source of BVDV from the herd and avoidance of the losses associated with the development of fatal MD.

iii) Artificial active immunization (vaccination).

Prevention of BVDV infection may not be guaranted with the vaccines that are currently available (Ernst *et al.*, 1983). Because of differences in the degree of immune responsiveness to various strains of BVDV, vaccines may have to contain more than one strain of the virus (Steck *et al.*, 1980). BVDV vaccines are currently available as either modified live or inactivated forms of virus (Baker, 1987).

a) Modified live viral (MLV) vaccines.

MLV vaccines for BVDV were first introduced in the late 1950s and most products contained the Oregon C24V isolate (Kahrs, 1981; Neaton, 1986). Immunity induced by MLV vaccine was regarded as being relatively solid and affording protection for the lifespan of most cattle. Serological studies indicated that virus neutralizing antibody titres in serum of naturally infected and vaccinated cattle persisted at least three years without evidence of decline (Kahrs *et al.*, 1966). MLV vaccines have the advantage of achieving high levels of immunity without the need for booster vaccination (Baker, 1987). However annual revaccination of cows does increase the titre of colostral antibodies and provides more protection for the newborn calf (Neaton, 1986).

Vacccination of young cattle which have preexisting neutralizing antibody titres of 1:100 or greater may not be efficacious due to neutralization of the vaccinal virus (Ernst *et al.*, 1983).

Vaccination against BVDV with MLV vaccine as a specific control procedure has proved to be controversial due to the questionable benefits and the possible risk of inducing MD or increased mortality due to respiratory disease in feedlot cattle (Martin *et al.*, 1982; Ruth, 1986). Epidemics of disease, often with clinical signs which were similar to MD have been reported following vaccination with MLV vaccines (Chennekatu *et al.*, 1967; Lambert, 1973; McKercher *et al.*, 1968; Rosner, 1968). The early MLV vaccines caused occasional MD which was seen 10 to 20 days after vaccination, as well as abortion in both vaccinated cattle and nonvaccinated herdmates (Lambert, 1973). With the use of a live vaccine the possibility of shedding of virus should also be considered (Baker, 1987). MLV vaccines retain all the foetopathic potential of wild virus types and their use at any stage during pregnancy is contraindicated (Liess *et al.*, 1984).

Possible causes of clinical disease following the use of MLV vaccine may be; unattenuated BVDV in the vaccine; incubation of BVDV in the host animal at the time of vaccination; occurrence of disease coincidental with vaccination; disease following vaccination but caused by a strain of BVDV other than that in the vaccine; immunosuppression induced by the vaccine strain of virus, resulting in secondary infection; and immunosuppression by stress at the time of vaccination, allowing the vaccinal strain of virus to cause disease (Baker; 1987; Neaton, 1986; Potgieter, 1988). However, the most important explanation for the occurrence of MD following live vaccine is that animals were persistently infected and developed MD when they became superinfected with the vaccinal strain of the virus (Bolin *et al.*, 1985; Brownlie *et al.*, 1984; Roeder and Drew, 1984).

A more recently developed MLV vaccine which incorporates a temperature sensitive strain of BVDV induces an antibody response and has not been associated with clinical signs or leucopaenia. Because of its restrictive temperature of growth, this vaccinal strain of virus did not cause foetal infection (Lobmann *et al.*, 1984). Stahl and others (1987) recommended administration of live virus vaccines to calves between six and ten months old to prevent intrauterine infection during their first pregnancy. However the

indications for vaccination must be clearly defined and vaccination programmes must be planned with an awareness of the epidemiological importance of the particular management system.

b) Inactivated BVDV vaccines.

An inactivated vaccine incorporating the Singer isolate was first introduced in 1982 (Neaton, 1986). Inactivated vaccines have the advantage of being safe for use in pregnant animals, and of not causing disease or immunosuppression. However the inactivated vaccines have the disadvantage of requiring revaccination to achieve protective immunity. Also the duration of immunity may not be as long as that attained with MLV vaccines. The necessary schedules of injection and other costs have precluded their wide usage in some countries (Littlejohns, 1988). Use of current inactivated vaccines requires that animals are revaccinated annually after being initially vaccinated twice between six and ten months of age (Ernst *et al.*, 1983; McClurkin *et al.*, 1984). The adujuvant generally used for inactivated virus is a form of aluminium hydroxide. Recently a new adjuvant " prolong " (Diamond scientific, USA) has been developed for use with an inactivated vaccine (Neaton, 1986). The vaccinated animals had antibody titres for at least six months after receiving a single two ml dose of " prolong " vaccine (Thomas and Jones, 1985).

Aim and scope of the present study.

A relatively high prevalence of SN antibody to BVDV in cattle in New Zealand, the occurrence of sporadic cases of MD, and the appearance of calves with some degree of congenital defects suggests that BVDV is ubiquitous in the cattle population in this country. However its maintenance and epidemiological features in dairy cattle is complex and requires closer study before specific control strategies can be developed.

The aim of this study was to investigate aspects of the epidemiology of the infection in a herd of dairy cattle, relating to the patterns of endemicity in different age groups within the herd and the possible ways in which infection is maintained within the herd. In an attempt to assess the maintenance of BVDV in commercial dairy farms under New Zealand conditions, one typical dairy farm with an average stock population was studied in detail. A large number of bobby calves, which were collected for slaughter in a local abattoir from several dairy farms in the Manawatu Region, were also examined.

CHAPTER TWO

GENERAL MATERIALS AND METHODS.

2.1 General materials and methods.

General materials and methods including the farm and methods applied and used in this study are described. Other specific methods are described in the relevant chapters.

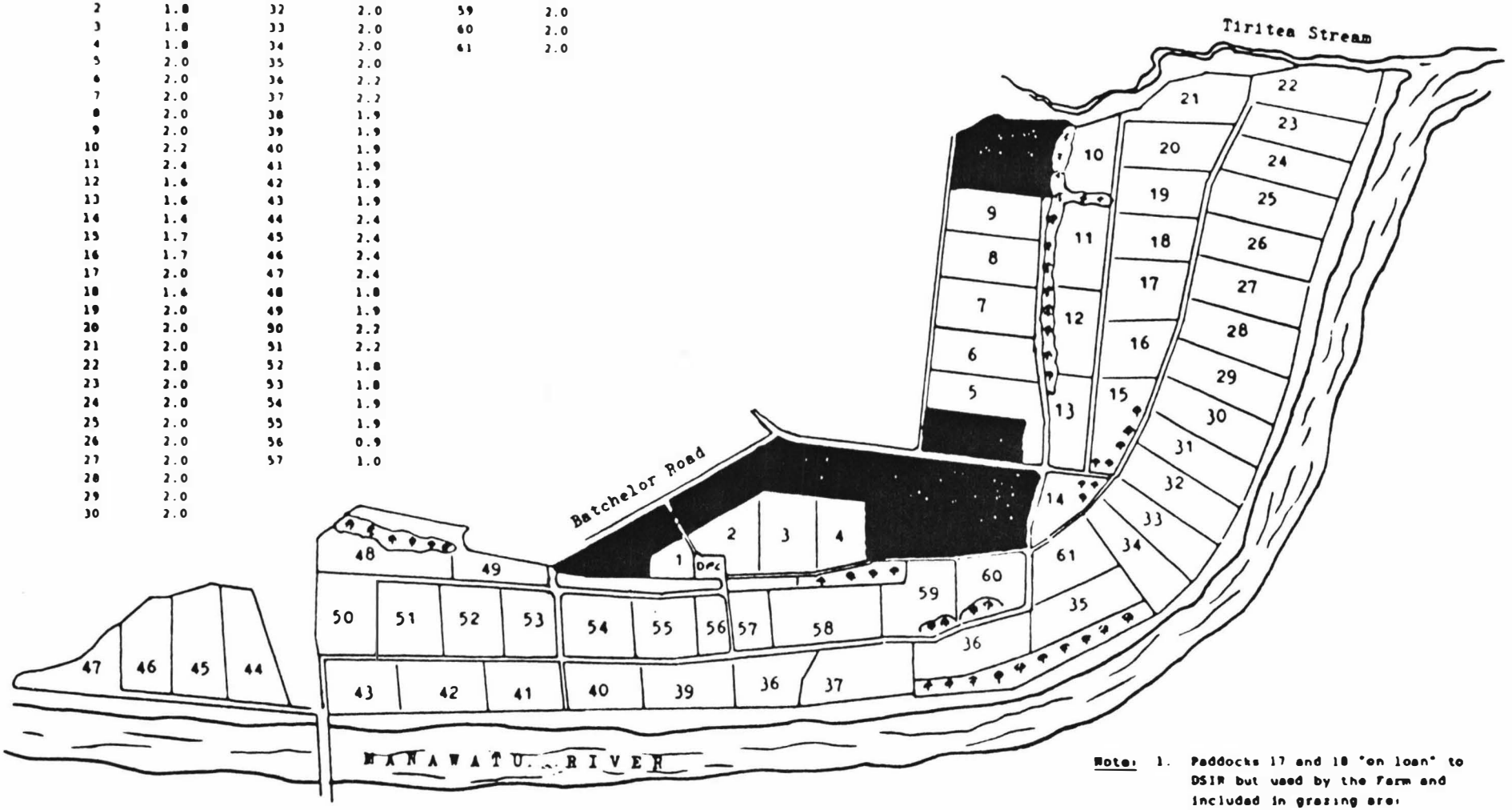
2.2. The farm.

The specific dairy farm under investigation was in the Manawatu Region and occupied 110 ha. of river flats which were subdivided into 61 paddocks (Fig. 2-1). It was operated commercially by Massey University on a town milk supply contract. The herd consisted of 330 Holstein-Friesian cattle. Replacement heifers were bred and reared on the property and bull calves were sold off the farm after rearing to eight weeks of age. There were two calving periods (autumn and spring) with the first-calf heifers being bred naturally at 15 months of age while artificial insemination (AI) was used with the adults. Bulls were introduced to the adult cows six weeks after AI had commenced and 15% of the adults calved to natural service. Rotational grazing was practiced throughout the year. Heifer calves born in autumn were kept together as one group and moved to fresh paddocks ahead of the milking herd so that they had no direct contact with older animals. Heifer calves born in spring were divided into smaller groups (five to ten per group) and were set stocked so that contact with older animals could occur when they shared the same paddock during the cycle of rotational grazing. Following natural service of the heifers at 15 months of age, they were kept together as a group, separate from the adult animals, until they calved and were introduced into the milking herd.

MASSEY UNIVERSITY

No. 1 Dairy Farm

Pdk No.	Area	Pdk No.	Area	Pdk No.	Area
1	1.2	31	2.0	58	2.9
2	1.8	32	2.0	59	2.0
3	1.8	33	2.0	60	2.0
4	1.8	34	2.0	61	2.0
5	2.0	35	2.0		
6	2.0	36	2.2		
7	2.0	37	2.2		
8	2.0	38	1.9		
9	2.0	39	1.9		
10	2.2	40	1.9		
11	2.4	41	1.9		
12	1.6	42	1.9		
13	1.6	43	1.9		
14	1.4	44	2.4		
15	1.7	45	2.4		
16	1.7	46	2.4		
17	2.0	47	2.4		
18	1.6	48	1.8		
19	2.0	49	1.9		
20	2.0	50	2.2		
21	2.0	51	2.2		
22	2.0	52	1.8		
23	2.0	53	1.8		
24	2.0	54	1.9		
25	2.0	55	1.9		
26	2.0	56	0.9		
27	2.0	57	1.0		
28	2.0				
29	2.0				
30	2.0				



Note: 1. Paddocks 17 and 18 "on loan" to DSIR but used by the Farm and included in grazing area.

Paddock 11 (loaned to DSIR - not used by the Farm; and

Paddock 44 taken for bridge construction.

Fig. 2 - 1. Diagram of Massey No. 1 dairy farm, shed and paddocks.

2.3 Animals sampled.

The prevalence of SN antibodies and viraemia with BVDV in a cross section of animals of different age was studied. Blood samples were collected from 161 of 182 (88.5%) adult milking cows, and 20 of 69 (28.9%) 12 months old heifers.

The group of 69 heifers was also tested again when they were 15, 16 and 24 months of age. At each time 20 animals were chosen from the group without any deliberate selection.

A cohort study was performed by sampling 21/25 female calves, born in autumn 1988, at one month intervals from one to four days of age up to 12 months of age. At the beginning of the blood collection from the cohort of calves their dams were also tested for comparison of maternally derived antibody titres.

2.4 Collection of blood samples from bobby calves.

Blood samples were collected from 58 bobby calves which were chosen without any deliberate selection from approximately 1000 bobby calves that had been presented for slaughter at a local abattoir. The samples were collected on the 8th and 9th of August 1988 during the peak calving period for the Manawatu Region. The age of bobby calves is theoretically not less than four days. Unfortunately it was not possible to know the precise age of these calves but it is unlikely that many were much older than four days.

2.5 Collection and processing of blood samples.

Blood was collected into ten ml evacuated glass tubes (Venoject, Terumo, Japan) and allowed to clot at ambient temperature for approximately 24 hours. The serum was removed by centrifugation at 600g for ten minutes, and a two ml aliquot was stored at -20^{o}C until tested. All sera were heat inactivated at 56^{o}C for 30 minutes before being tested.

Each blood clot was disrupted by extruding through a sterile ten ml syringe into two ml of phosphate buffered saline (PBS) pH 7.2 and stored in a five ml vial (Sterlin, Feltham, England) at -70^{o}C until used for virus isolation.

2.6 Cell culture technique.

2.6.1 Propagation and maintenance of cell monolayer.

Madin Darby bovine kidney (MDBK) cell line (Obtained from ICI, Coopers Animal Health N.Z. Ltd., Lower Hutt) was used for propagation of BVDV and for SN tests. The cells were extensively tested for the presence of adventitious BVDV by viral interference assay (VIA) and fluorescent antibody test (FAT) with fluorescein conjugated anti-BVDV anti-serum before any assay was carried out.

The MDBK cell cultures were routinely propagated by seeding cells at a concentration of 1×10^5 cells/ml into 75 cm^2 plastic tissue culture flasks (Cat. No. 1-537332, Nunclon, InterMed, Denmark) in 20 ml volumes of growth medium which consisted of Minimum Essential Medium (Eagle's modified) (Cat. No. 10-101-20, Flow Laboratoris, U.K.) with the addition of 10% heat inactivated (56^oC for 30 minutes) horse serum (Cat. No. 200-605AJ, Gibco N.Z. Ltd., Auckland),1% of antibiotic solution (penicillin 100 units/ml, streptomycin 10 mg/ml) and 1% vitamin solution (Cat. No. 10K4651, Gibco N.Z. Ltd., Auckland) (PSK), and incubated at 37^oC. Confluent monolayers were attained and the cells were subpassaged every five days and used at passage levels between 125 and 150. For maintenance of the cell monolayers the horse serum concentration was reduced to two per cent v/v.

All work with the cell cultures was carried out in a laminar flow cabinet (Model No. CF 435, Gelman Clemco PTY. Ltd., Artarmon, N.S.W. Australia) using aseptic techniques in a room reserved exclusively for this purpose. Media,tissue culture flasks, microtitre plates and glassware were sterilized by ultrafiltration, gamma irradiation or by autoclaving as appropriate.

2.6.2. Trypsinization of confluent monolayers.

Growth medium was decanted by suction and monolayers were washed gently two times with ten to 15 ml of sterile phosphate buffered saline (PBS) pH 7.2 (Appendix I). Trypsinization was carried out with two ml of 0.05% trypsin solution in antibiotic-trypsin-versene (ATV) (Appendix I) mixture for two minutes at 37^oC. The flasks were shaken vigorously to separate aggregates of cells before the addition of eight ml of growth medium. The suspended cells were dispensed either into tissue culture flasks or 96-well

microtitre plates (Cat.No. 1-67008, Nunclon, InterMed, Denmark) at $1x10^5$ cells/ml of growth medium. The cells were kept in suspension by gentle shaking for two to three seconds and a 0.2 ml aliquot was removed and added to 1.8 ml of trypan blue solution (Appendix I). After thorough mixing, a volume of this solution sufficient to fill both chambers of Neubauer haemocytometer, was taken and the mean of the unstained viable cells in the four large squares was determined. The number of cells in the original suspension was then calculated.

2.6.3. Preparation of primary cell cultures of bovine foetal lung.

Bovine foetal lung (BFL) primary cell cultures were established as described by Goldsmit and Barizilai (1975).

Bovine foetuses (30 to 50 cm long) at approximately three to four months of gestation were obtained from a local abattoir. The foetuses were transported to the laboratory in plastic bags and the lungs were removed aseptically within one hour of removal of the foetuses from their dams. Procedures for primary BFL cell culture preparation were as follows:

i) A portion of the lung was removed aseptically and placed in a sterile Petri dish. In the laminar flow cabinet the tissue was chopped finely with crossed scalpel blades and fragments of connective tissue were separated.

ii) The tissue was then transferred to a trypsinizing flask and washed three times with PBS containing 2% PSK or until the supernate was clear. The tissue was allowed to stand in the last wash for three to five minutes at room temperature (20^oC).

iii) Trypsinization was performed by adding freshly prepared trypsin solution (0.4% of w/v in PBS) (Appendix I) and gently stirring for 15 minutes at 37^oC. Coarse debris was removed by filtration through a sterile metal sieve in a funnel and the filtrates were collected into sterile centrifuge tubes containing one ml of horse serum for every ten ml of the filtrate. One or two further 30 minute trypsinizations and collections were made to increase the cell yield.

iv) Following centrifugation (500g for ten minutes),the pellet of cells was suspended in one ml of modified growth medium and shaken gently. Nine ml of the same medium was added and cell counting was performed as described in section 2.6.2. The growth medium, consisted of minimum essential medium Eagles modified (Cat. No. 10-101-20, Flow Laboratories, U.K.), 15% horse serum which was heat inactivated at $56^{o}C$ for 30 minutes (Cat. No. 200-605AJ, Gibco, N.Z. Ltd., Aucland), 2% antibiotic solution (penicillin 100 units/ml, streptomycin 10mg/ml, kanamycin 10mg/ml, 2% vitamin solution (Cat. No. 10K 4651, Gibco, N.Z. Ltd., Auckland) and 10% solution of tryptose phosphate (Difco Laboratories, Detroit, Michigan, USA).

v) The cell suspension was further diluted in growth medium to obtain a final concentration of $2x10^5$ cells/ml and dispensed into 75 cm^2 tissue culture flasks in 20 ml volumes. Flasks were incubated at $37^{o}C$ and the medium was replaced at 48 hours after seeding.

vi) The cells were examined under the phase contrast microscope (Olympus optical Ltd., Tokyo, Japan) at ten or 20X magnifications for any cellular degeneration, rounding or vacuolization. Flasks from each passage level were incubated and examined every 24 hours upto seven days. At the fourth passage level the cells were tested by VIA and direct FAT for the presence of any noncytopathic strain of BVDV. Details of the procedures of both tests are described in 2.8.3 and 2.8.4. Batches of cells which gave negative results in the above tests were subpassaged in 175 cm^2 tissue culture flasks (Cat. No. 1 56502, Nunclon, InterMed, Denmark) prior to storage.

vii) At the fifth passage level the cells were trypsinized and counted as already described. The cells were suspended at a concentration of $2x10^5$ cells/ml in growth medium and centrifuged (500g for ten minutes). The supernatant was removed and the cell pellet obtained from each 175 cm^2 flask was resuspended in four ml of storage medium (70% Minimum Essential Medium, 20% horse serum and 10% dimethyl sulphoxide (DMSO)). The mixture was transferred in one ml volumes to screw capped plastic ampoules, labelled, placed in an

insulated box and stored at 4°C for one hour, at -20°C for one hour, at -70°C for four hours and in liquid nitrogen (-196°C) for long term storage. The estimated rate of cooling was one degree Celsius per ten minutes.

When required, the cells were thawed at 37°C in a water bath (two to three minutes) and transferred to one ml of growth medium, mixed and transferred to eight ml of the same medium. The viable cells were counted as described and subpassaged in the same manner as indicated in section 2.6.2. Confluent monolayers were observed after four days and further passaging was conducted as required. Monolayers of BFL cells were used for isolation of viruses between passage levels seven and ten.

2.7. The serum neutralization (SN) test.

2.7.1. The cell culture.

The MDBK haploid cell culture was used for the propagation and titration of the stock virus and also for SN test procedures.

2.7.2. Propagation and storage of the stock virus.

The NADL strain, a cytopathic biotype, of BVDV was obtained from the Wallaceville Animal Health Laboratory, Lower Hutt. Working stock was prepared by passage of this virus in MDBK cells. One ml of the NADL viral suspension as received was inoculated onto an MDBK monolayer and incubated for one hour at 37°C. Then 20 ml maintenance medium (same as growth medium except the horse serum concentration was reduced from ten per cent to two per cent in volume) was added and the cells were further incubated at 37°C. Within four days a CPE was observed to involve 80% of the monolayer and the cells were frozen and thawed once. This suspension was designated stock virus, and was distributed in one ml volumes in bijou bottles and stored at -70°C until required.

2.7.3. Titration of the stock virus.

The titre of the virus in cell culture was determined by serial tenfold dilution of the stock virus suspension in maintenance medium. This was performed in 96 well tissue culture micro-titre plates in 100 ul volumes per well,

from 10^{-1}, to 10^{-9} with four replicates of each dilution. The micropipette tips were changed between dilutions. Similar volumes of cell suspension were added and the plate was covered and incubated in a five per cent CO2 atmosphere at 37^{o}C for five days. Control, uninfected cells were also included in the same plate. The end point of the dilution series was recorded as the highest dilution exhibiting CPE with total or partial degeneration of the infected cells. The fifty per cent end point titre (CCID50) was calculated by the Karber-Spearman method (Lennette and Schmidt,1979).

2.7.4. The SN test procedure.

Twofold serial dilutions of sera from 1:2 to 1:2048, were made in 50 ul volumes of maintenance medium in 96-well tissue culture micro-titre plates and equal volumes of virus suspension, containing 100 CCID50 were added to each serum dilution. The virus serum mixture was held for 90 minutes at room temperature before 100 ul of growth medium containing 1x10^{5} cells was added to each well. The plates were incubated in a 5% CO2 atmosphere at 37^{o}C for five days. Controls included with each test were reference positive and negative sera, titration of the virus, test serum toxicity for cell culture controls (test serum plus cells but without virus) and cell control. The titre of each serum was taken as the reciprocal of the highest dilution of test serum which completely inhibited the CPE of the added virus. Sera which did not inhibit the CPE of virus at a final 1:4 dilution were considered as negative. Evaluation of the precision of this test indicated that results of repeated tests with reference positive sera were always within one dilution to the right or left of the mean value.

2.8. Isolation of BVDV from blood clots.

2.8.1. The cells.

BFL cell cultures were used for all attempts to isolate virus from blood clots. Low passages (7-10) were used for inoculation of samples. The cells were grown in 175 cm^{2} tissue culture flasks. The cell monolayer was trypsinized, counted and dispersed in modified growth medium as described. Cells at a concentration of 2x10^{5}/ml were distributed in 48-well tissue culture plates (Cat. NO. 3548, CoStar, Cambridge) in one ml volumes per well using a ten ml pipette. The plates were then covered and incubated in a 5% CO2 atmosphere at 37^{o}C. After three days, confluent monolayers were attained.

2.8.2. Inoculation of the sample.

The growth medium was removed from the plates by suction and 200 ul of each blood clot which has been stored at -70^{o}C was added gently to a pair of wells and incubated at 37^{o}C for one hour. Then 0.8 ml of maintenance medium (same as modified growth medium for BFL cell culture but with the horse serum concentration reduced to 5% v/v) was added and the plates were covered and incubated in a 5% CO2 atmosphere at 37^{o}C. Cell controls (without addition of sample) were also incubated in the same manner. After three days the plates were examined under a phase contrast microscope at 10x magnification (Olympus Optical Ltd., Tokyo, Japan) for possible CPE. The plates were then frozen and thawed once and 200 ul amounts from each well were transferred to fresh monolayers and similarly treated. The procedure was repeated one further time. Cell cultures which failed to show CPE after three passages were considered as negative for cytopathic BVDV. Cultures which were negative for CPE were tested for the presence of noncytopathic virus by VIA using the NADL strain of BVDV.

2.8.3. Viral interference assay procedure.

The medium was removed by suction and one of each pair of wells was challenged by adding 50 CCID50 of the NADL strain in a 200 ul volume. The plate was incubated at 37^{o}C for one hour and 0.8 ml of maintenance medium was then added. Each test procedure included test sample inoculum (challenged and unchallenged as only one of the two rows was challenged with NADL), control cells inoculated only with challenge virus (but not with test sample fluid), control cells (without any inoculum) and challenge virus titration.

Cultures were considered as infected with noncytopathic virus when complete CPE was induced by the NADL strain in inoculated control cell cultures but was suppressed or inhibited (with at least 20% interference) in the test culture. The cultures which were positive by VIA were collected in bijou bottles and stored at -70^{o}C for further testing by FAT. Some of the VIA negative cultures were also tested by FAT.

2.8.4. Direct fluorescent antibody test (FAT).

The gammaglobulin fraction of a batch of foetal calf serum which had an anti-BVDV SN antibody titre of 2.4 (Log10) had been prepared and

conjugated with fluorescein isothiocyanate by standard procedures. This preparation gave specific fluorescence with cell cultures which were infected with cytopathic or noncytopathic strain of BVDV but not with uninoculated cells or with cells infected with infectious bovine rhinotracheitis virus or with PI-3 virus.

Leighton tubes with coverslips were washed with 10% HCl, rinsed with distilled water, dried at 37^{o}C and sterilized by autoclaving.

Two ml volumes of low passage (passage level eight or nine) BFL cell suspension containing 10^5 cells/ml in modified growth medium were added to Leighton tubes with coverslips. At three days post inoculation confluent monolayers were observed and the medium was removed by suction. Test samples or the NADL strain (50CCID50) were added in 200 ul volumes and the tubes incubated at 37^{o}C for one hour. Then 1.8 ml of maintenance medium was added and the tubes were sealed air tight and incubated at 37^{o}C for 36 hours or until the first sign of cytopathic effect in the positive control was evident. Coverslips with noninfected cultures were also incubated in the same manner.

The cultures on the coverslips were fixed with two ml of cold acetone at 4^{o}C for ten minutes. The acetone was removed by suction and the coverslips were gently rinsed with distilled water and air dried. They were then flooded with a 1:10 dilution in PBS of the fluorescein-conjugated anti-BVDV antiserum for 30 minutes at room temperature (20^{o}C). The slides were then washed in two changes of PBS pH 7.2 for 30 minutes with constant stirring. Finally they were rinsed with distilled water, air dried, and were mounted on a glass slide with equal volumes of glycerine and double strength of PBS pH 7.2.

Slides were examined using a fluorescent microscope (Olympus Optical Ltd.,Tokyo, Japan with a darkfield condenser, illuminated by halogen bulb 12V 100WHA-L, with light filter KB-4). A 10X and/or 20X magnification was used to observe the field and individual cells as required. Positive cell cultures showed clear cytoplasmic fluorescence while the negatives showed no evidence of fluorescence.

CHAPTER THREE

Serological comparisons of New Zealand isolates of BVDV with the NADL strain.

INTRODUCTION.

The results of reported serological studies indicate that various strains of cytopathic and noncytopathic strains of BVDV are antigenically related (Castrucci *et al.*, 1975; Gillespie *et al.*, 1961; Peters *et al.*, 1986; Wensvoort *et al.*, 1989). Common serum neutralizing, complement fixing and gel diffusion precipitating antigens have been demonstrated in various strains of BVDV (Magar *et al.*, 1988). Antigenic differences have also have been reported to exist (Fernelius *et al.*, 1971; Itoh *et al.*, 1984; Peters *et al.*, 1986). These were demonstrated by relatively lower antibody titres in SN tests, variations in intensity of fluorescence and by panels of monoclonal antibodies.

Persistent infection and immunotolerance appear to be strain specific (Bolin *et al.*, 1985). While persistently infected cattle are tolerant to the specific strain of BVDV with which they are infected, they are capable of developing low titres of neutralizing antibody in response to infection with heterologous strains of the virus (Brownlie *et al.*, 1987). Both cytopathic and noncytopathic biotypes, which are antigenically related, are involved in the pathogenesis of MD (Howard *et al.*, 1987). Not all persistently infected cattle develop MD when superinfected with cytopathic BVDV experimentally (Corapi *et al.*, 1988) and it is concluded that, for MD to occur, the noncytopathic and the cytopathic strains must be antigenically very similar.

The present investigation was conducted to determine the serological relationship between four New Zealand isolates of BVDV and the NADL strain. The NADL strain was used as the antigen in SN tests conducted on cattle sera for the epidemiological study. If New Zealand isolates of BVDV were only distantly related antigenically then the use of the NADL strain could yield a large number of false negative results.

Materials and methods

The cell culture.

MDBK cells at passage level 138 to 140 were used in this study. Details of the cell culture procedure are described in chapter two.

The viruses.

The NADL strain (obtained from Wallaceville Animal Health Laboratory, Lower Hutt), strains 109, 510, 709 (noncytopathic strains isolated from three calves with cerebellar hypoplasia) and the New Zealand cytopathic strain designated as "BVD-MDBK" were already available in the laboratory. Direct titration was possible only for the NADL and BVD-MDBK strains which had titres of $2x10^7$ CCID50/100ul and $2x10^6$ CCID50/100ul respectively.

The sera.

i) Rabbit anti-BVDV anti-serum. Rabbit antisera against NADL and two of the noncytopathic strains (109,510) had been raised in rabbits by giving them intramuscular injections of each virus, which had been propagated in MDBK cultures and blended with an equal volume of Freund's complete adjuvant.

ii) Bovine sera. Four bovine sera, which were positive in the SN test against NADL virus, were used in the cross neutralization test. Two of these sera had high titres (3.3) and the other two had low titres (0.6 and 1.8). The reference positive and negative sera which were used throughout the epidemiological investigation were also included.

iii) Pig sera. Pig serum against strain 709 was raised by intranasal inoculation of 2 ml of viral suspension (approximate titre 10^7 CCID50/ml). Serum was prepared from blood collected 28 days post inoculation.

SN test.

All sera were heat inactivated at 56^oC for 30 minutes. Serial twofold dilutions of each serum, were distributed, in 50 ul volumes, in the wells of 96-

well microtitre plates. Equal volumes of viral suspension, containing 100 CCID50, were added to each well and the plates were incubated at room temperature for 90 minutes.

Rabbit antisera to strains NADL, 109, and 510, pig antiserum to strain 709 and the four bovine sera were tested against strains NADL and BVD-MDBK.

A 100 ul volume of growth medium containing 10^5 cells/ml of MDBK was added to each well and the plates were incubated in 5% CO2 in air at 37^oC for five days. SN test procedures, cell controls, titration of the viruses and other procedures were carried out as described in chapter two.

RESULTS.

The results are summarized in Table 3-1. For each of the sera, the reactions with the NADL strain of BVDV are similar to those with the BVD-MDBK strain. Any difference in titres obtained with one serum against the two viruses was never more than two steps in dilution. Three of the four bovine sera gave identical reactions with each of the two viral strains. The two rabbit antisera against the noncytopathic strain 109 and 510 gave slightly higher titres against BVD-MDBK than against NADL but these were still within two dilutions.

TABLE 3-1.

Neutralization of strains of NADL and BVD-MDBK by rabbit and pig anti-serum raised against NADL, 109, 510 and 709 strains, and also by bovine serum collected from the dairy farm.

	Antiserum							
	Rabbit			Pig	Bovine			
Virus	NADL	109	510	709	20	251	21	134
NADL	2.7*	2.1	2.7	0.9	3.61	1.8	0.6	3.31
BVD-MDBK	2.4	2.7	3.31	NT	3.61	1.8	< 0.6	3.31

* = antibody titre (log10)

NT = not tested

DISCUSSION

The results of the current study with rabbit and pig antisera, and also with bovine sera, which were obtained from the dairy farm, showed that the virus (or viruses) that elicited SN antibody production in the cattle population in New Zealand is serologically very similar to the NADL strain of BVDV. In other studies SN antibody titres ranging from 0.6 to 3.3 were found in pig sera collected from the field and tested against the NADL strain (Shu personal communication, 1989).

Because of the cytopathogenicity of the NADL strain in a wide range of cell cultures of bovine origin, and the availability of both the virus and sensitive cell cultures in the laboratory it was decided to use the NADL strain as stock virus for the detection of SN antibody, and as a challenge virus in viral interference assays for viral detection. This study provided additional information, supporting the hypothesis that NADL was appropriate to use as the challenge virus for serological and virological studies on the prevalence and incidence of infection with BVDV in New Zealand. Strains of BVDV, which were used in the previous investigations on the prevalence of SN antibodies in bovine sera in New Zealand were C60F and the vaccine strain of Bovax (Fastier and Hansen, 1966; Robinson, 1971).

Antisera raised by rabbit inoculation and tested using the viruses, NADL and BVD-MDBK, indicated that they were antigenically closely related. The NADL strain was also neutralized by pig serum raised against the 709 strain of BVDV. It was not possible to determine the cross neutralization reactions using noncytopathic viruses, because these viruses require methods other than a direct SN test to determine antigenic relationships. The variations in titres between the NADL and BVD-MDBK strains of BVDV were not more than two dilutions, which were within the expected range of reproducibility of the SN test. Various strains of cytopathic and noncytopathic biotypes of BVDV are antigenically related (Castrucci *et al.*, 1975; Gillespie *et al.*, 1961; Peters *et al.*, 1986).

The results of this study showed that various strains of cytopathic and noncytopathic biotypes of BVDV isolated in New Zealand are serologically related to the NADL strain of BVDV. Determination of how close the antigenic relation between various strains of BVDV requires further investigation.

SUMMARY

i) The results of cross neutralization tests using antisera raised against different strains of BVDV, which were isolated in New Zealand, and the NADL strain indicated that they were antigenically closely related. Therefore it is appropriate to use the NADL strain of BVDV in further studies on the prevalence of SN antibodies and for viral detection in cattle and other animals in New Zealand.

ii) Identification and characterization of the strains of BVDV is important if control is considered by immunization. It may also have epidemiological significance in identification of persistently infected animals with complete or incomplete immunotolerance.

CHAPTER FOUR.

Serological studies on the epidemiology of infection with BVDV in a dairy herd.

INTRODUCTION.

The SN test has been used to conduct serological investigations for anti-BVDV specific antibody in cattle sera (Gillespie *et al.*, 1961). The SN test is an ideal test for the detection of neutralizing antibodies to specific antigens of diseases associated with viruses. Since most primary postnatal infections with BVDV are subclinical the SN test has important diagnostic applications. The prevalence of SN antibody in the herd is an indication that the animals were exposed to the virus at sometime during their life. SN antibody is detectable in serum at two to four weeks post infection, and may provide immunity lasting for several years (Barber *et al.*, 1985). Being antibody negative, however, does not necessarily mean that the animals have never been infected with the virus. Antibody negative animals may be persistently infected and sources of infection to animals in close contact, thus maintaining the infection in the herd. Animals infected recently may also give negative results before their serum antibody reaches detectable titres. Fully susceptible animals are also antibody negative.

Although the SN test by itself may provide substantial information about the epidemiological status of the herd in terms of BVDV infection, virus isolation and tests of paired sera three weeks apart may be necessary if a specific control strategy is to be implemented.

The other feature to be considered is the presence of maternally derived antibody in calf sera. Calves may acquire SN antibodies from their dams (Coria and McClurkin, 1978; Kendrick and Franti, 1974) and therefore the interpretation of SN antibody titres in young calves is complicated due to the presence of maternally derived antibody for some months after birth.

The sensitivity and specificity of the SN test has been determined by comparing the test with *in vivo* inoculation of calves with the virus and the SN

test has been shown to be a good indicator of the presence of antibody against BVDV in bovine serum (Robson *et al.*, 1960).

In the present study the SN test has been standardized locally to suit the available strain of virus (NADL) and the cell culture in this laboratory. The reproducibility of the test has been evaluated by repeated tests with reference positive control serum, which were conducted with every batch of tests.

The epidemiology of BVDV was studied in one dairy farm by measuring specific SN antibodies in groups of animals described in section 2.3 and 2.4.

Materials and methods.

i) The farm, the animals, sample collection and standard SN procedures have been described in chapter two.

ii) Statistical analysis of serological data. The serological data of each age group were compared in a one way analysis of variance and F test using the PANACEA computer programme (Pan Livestock services Ltd., Department of Agriculture, University of Reading , England). For all tabulations and calculations the titres were converted to log10 values.

RESULTS

Cross-sectional observations.

SN antibody to BVDV was detected in the sera of all but four animals aged from 12 months to more than eight years (Table 4-1 and Fig.4-1). One antibody negative animal was three years old, two were four years old and one was eight years old. Three bulls introduced to the farm for natural mating, which were tested at the time of introduction to the farm and one month later, had antibody titres of 3.01, 2.4 and 2.4 respectively in both tests. There appeared to be a slight increase with age in the geometric mean titres of the groups (Table 4-1) and more animals had higher titres in the older groups of animals up to the age of six to seven years and then a slight decline was observed (Fig.4-1) . However the distribution of anti-BVDV antibody titres in the groups of replacement heifers and adult cows were not significantly

different ($p > 0.05$). By chance, 16 of the replacement heifers were bled and tested on several occasions from 12 to 24 months of age (Table 4-2). For most of these animals the antibody titre did not change significantly with time (i.e., within the reproducibility of the test). However five animals had greater than four fold rises in titre (numbers 3,5,7,8 and 10).

TABLE 4-1.

Serum neutralizing antibody to BVDV in heifers and cows from the same farm.

Category	Age (years)	Number tested	Number (%) positive *	Geometric mean titre (SD)
heifer	1	20	20 (100)	2.37 (0.63)**
heifer	1.25	20	20 (100)	2.58 (0.73)
heifer	1.3	20	20 (100)	2.64 (0.75)
heifer	2	19	19 (100)	2.61 (0.41)
cow	2-3	78	77 (98.7)	2.70 (0.54)
cow	4-5	52	50 (96.0)	2.79 (0.67)
cow	6-7	15	15 (100)	2.97 (0.35)
cow	$\geq$ 8	16	15 (93.8)	2.95 (0.54)

*positive = SN antibody titre of $\geq$ 0.6

** Log10 SN antibody titre.

Fig. 4-1

Distribution of serum neutralizing antibody titres against BVDV in heifers and cows on one farm.

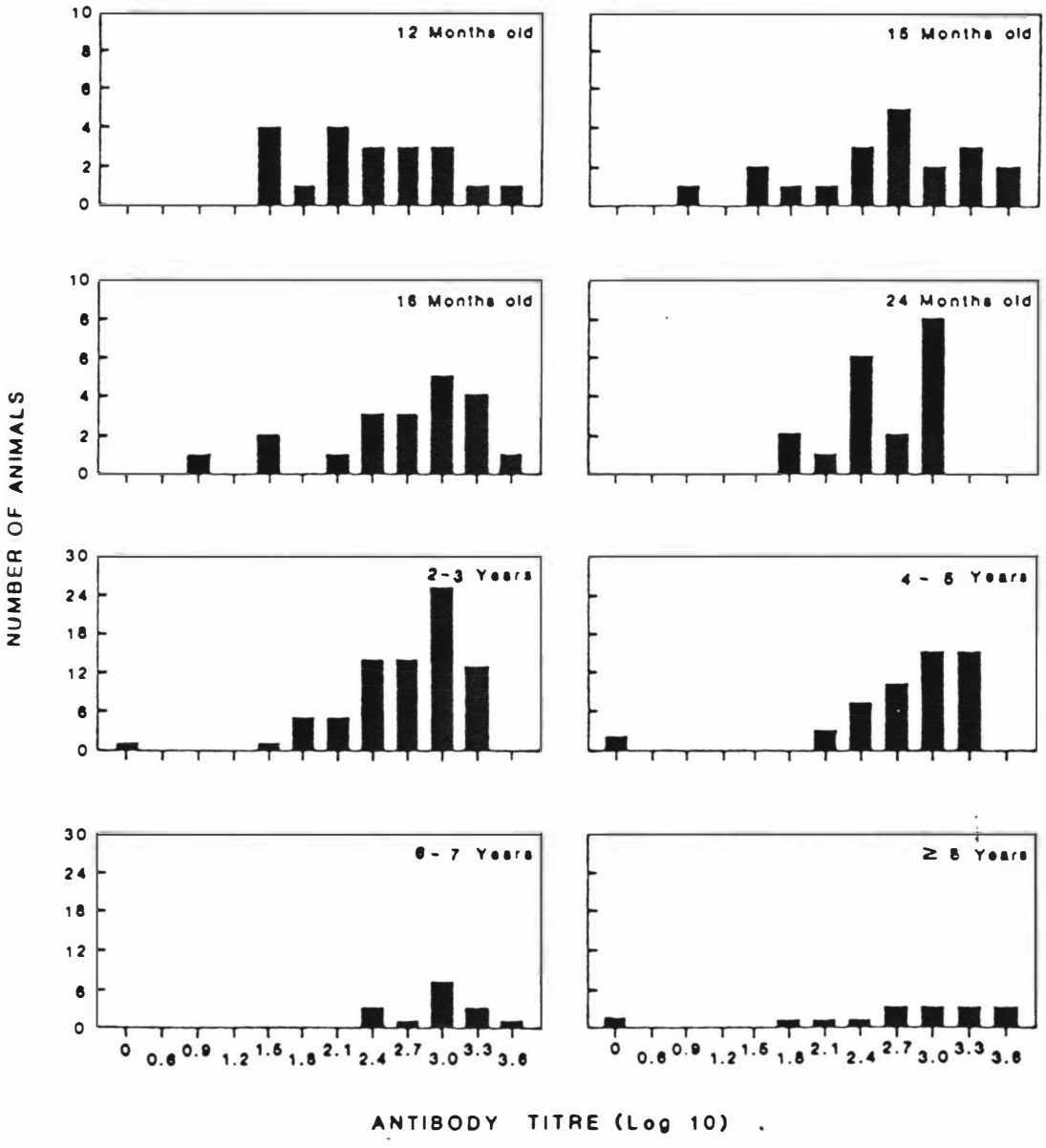

12 Months old
15 Months old
18 Months old
24 Months old
2-3 Years
4 - 5 Years
6 - 7 Years
≥ 8 Years
NUMBER OF ANIMALS
ANTIBODY TITRE (Log 10)
0 0.6 0.9 1.2 1.5 1.8 2.1 2.4 2.7 3.0 3.3 3.6

TABLE 4-2.

Serum neutralizing antibody to BVDV in a group of heifers between 12 and 24 months of age.

	Age at time of blood collection (months)			
Heifer number	12	15	16	24
1	2.70*	2.70	NT**	3.01
2	2.40	2.70	3.01	NT
3	2.10	NT	2.40	3.01
4	2.10	2.40	2.70	NT
5	2.40	3.61	3.61	NT
6	2.70	3.01	NT	NT
7	NT	0.90	0.90	3.01
8	NT	1.50	NT	2.40
9	3.61	3.61	NT	NT
10	2.10	NT	3.01	NT
11	2.10	NT	2.40	NT
12	1.80	1.80	NT	2.40
13	3.31	3.31	3.31	3.01
14	NT	2.40	2.70	3.01
15	2.40	NT	2.40	NT
16	1.50	2.10	2.10	Nt

* Log10 SN antibody titre.

** NT = not tested

Cohort of calves.

Twenty of 21 calves tested one to four days after birth had titres of SN antibody within four serial dilutions of their dams (Table 4-3). One calf remained antibody negative throughout the period of testing. The antibody titres of the calves declined steadily so that by the age of eight months all calves were negative and remained so up to 12 months (Fig.4-2). The geometric mean titre of the cohort at each monthly bleeding was plotted against age and the calculated regression line drawn (Fig.4-3). The half life of specific passive anti-BVDV antibody was calculated using the linear regression formula, as follows: $y = b + mx$ (where y = Log10 titre of passive antibody, x = time (in days) when tested, b = intercept at the y axis, and m the slope of the line) was $y = 2.5711 + (-0.3741)x$. In this study the half-life was 25 days.

TABLE 4-3.

Serum neutralizing antibody to BVDV in the cohort of 1-4 day old calves and their dams.

Calf number	Serum neutralizing antibody titre (Log 10).	
	Calf	Dam
1	2.70	3.31
2	3.01	3.31
3	3.31	3.01
4	2.10	2.40
5	2.10	3.31
6	3.61	3.61
7	3.61	3.31
8	3.01	3.01
9	3.61	3.31
10	2.40	3.31
11	< 0.60	3.31
12	3.31	3.31
13	3.01	3.31
14	3.61	3.31
15	3.31	3.61
16	3.01	3.31
17	3.61	3.31
18	2.40	3.01
19	3.01	3.31
20	2.10	3.01
21	3.01	3.01
Geometric mean titre	2.85	3.22
Standard deviation	0.83	0.25

Fig. 4-2.

Distribution of serum neutralizing antibody titres against BVDV in a cohort of 21 calves.

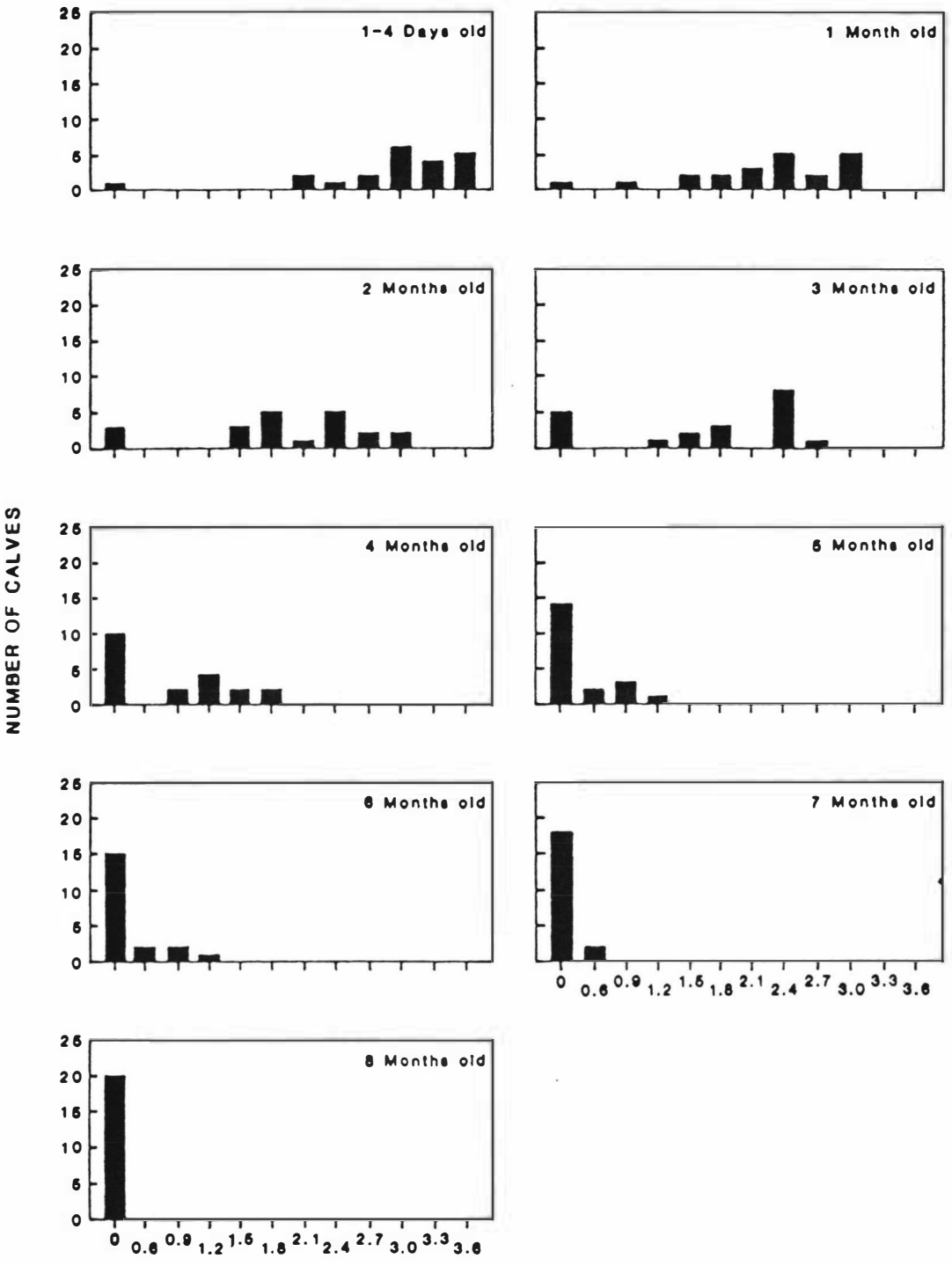

1-4 Days old
1 Month old
2 Months old
3 Months old
4 Months old
5 Months old
6 Months old
7 Months old
8 Months old
NUMBER OF CALVES
ANTIBODY TITRE (Log 10)
0
0.6
0.9
1.2
1.5
1.8
2.1
2.4
2.7
3.0
3.3
3.6

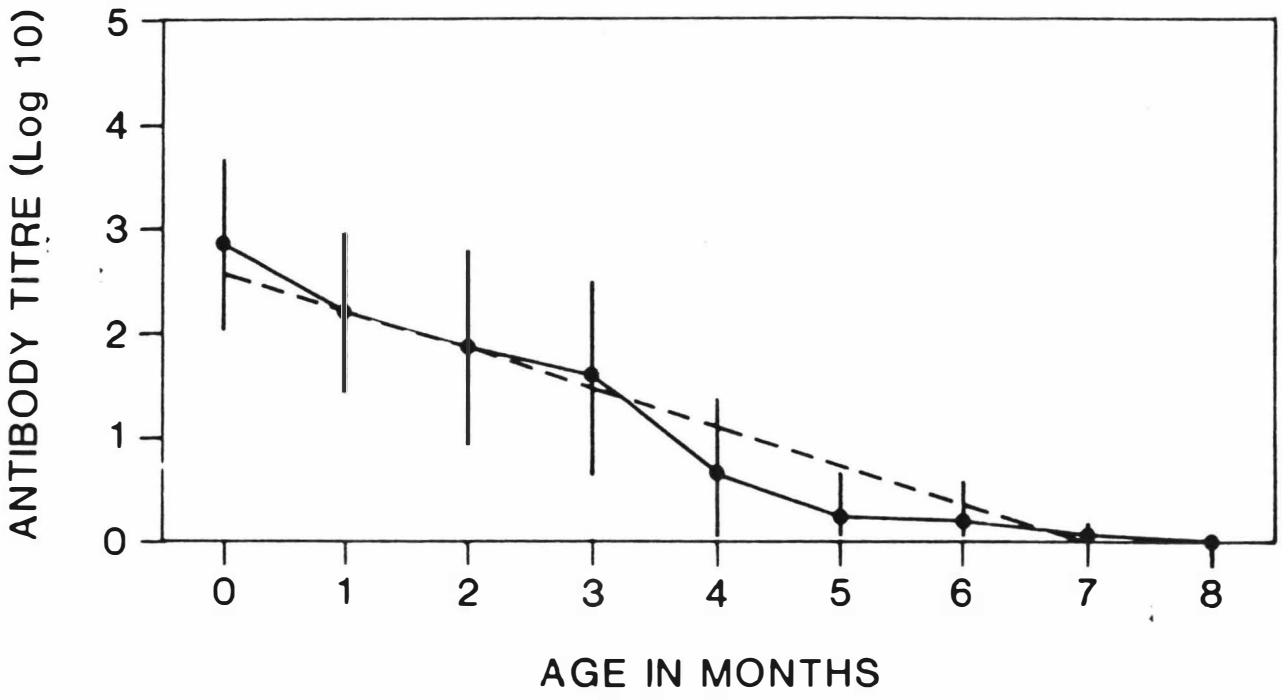
Fig. 4-3.

Serum neutralizing antibody to BVDV in a cohort of 21 calves.

0 = geometric mean titre

vertical lines represent one standard deviation.

$$Y = 2.57 + (- 0.3741) X$$

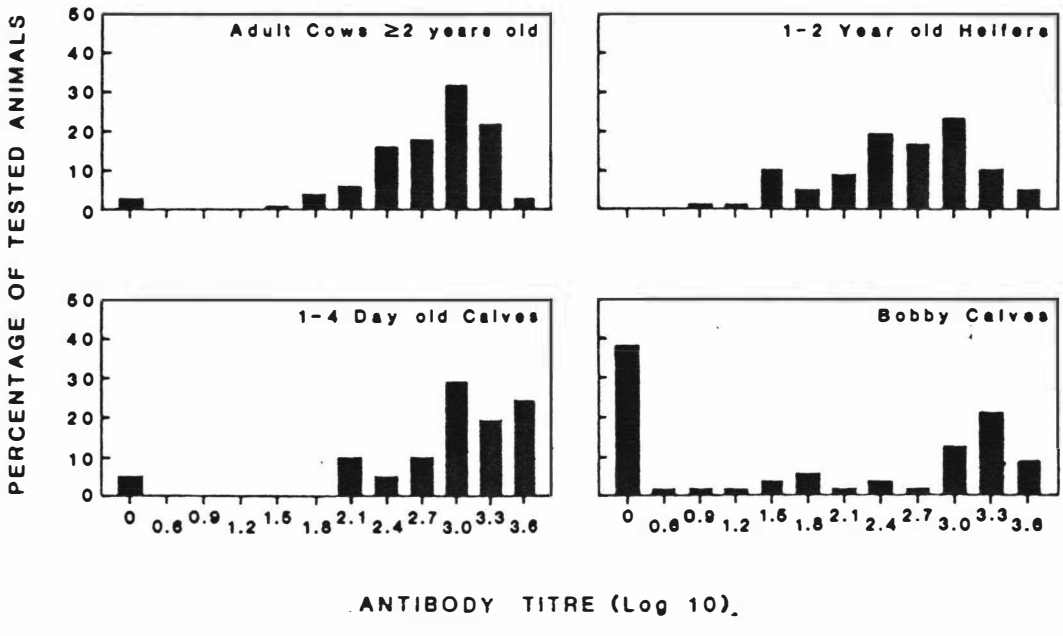

ANTIBODY TITRE (Log 10)
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
AGE IN MONTHS

Bobby calves.

Of the 58 blood samples from bobby calves at the local abattoir only 36 (62%) had detectable SN antibodies against BVDV. The range of titres was the same as that for groups of animals sampled on the farm (< 0.6 to 3.6), however many more bobby calves had low titres or were negative (Fig.4-4). The difference in distribution of SN antibody titres between the bobby calf group and calves or adults in the farm was highly significant ($p < 0.001$).

Fig. 4-4.

Distribution of serum neutralizing antibody titres to BVDV in groups of cattle on the farm and bobby calves at an abattoir.


Adult Cows ≥2 years old
1-2 Year old Heifers
1-4 Day old Calves
Bobby Calves
PERCENTAGE OF TESTED ANIMALS
0
10
20
30
40
50
0 0.6 0.9 1.2 1.5 1.8 2.1 2.4 2.7 3.0 3.3 3.6
ANTIBODY TITRE (Log 10)

DISCUSSION.

Because of its high immunological specificity the SN test is often the standard against which the specificity of other serological procedures are evaluated (Ballew, 1986). Sensitivity and specificity of SN tests have been evaluated, and the SN test for BVDV is an exellent indicator of prior infection with, and immunity to, BVDV (Robson *etal.*, 1960). Neutralizing antibody prevents BVD viraemia and plays a critical role in limiting primary BVDV infection (Shope *et al.*, 1976). A SN titre of 0.6 or greater has been regarded as indicating either a previous encounter with BVDV or passive immunity (Lambert, 1973; Malmquist, 1968).

In the cross sectional study 97.1% of the replacement heifers and adult cows were found to have antibody to BVDV. This is higher than the prevalence found in two earlier studies carried out in New Zealand which detected antibody in 41 and 66% of the animals which were surveyed (Fastier and Hansen, 1966; Robinson, 1971). The prevalence of serum antibody in the herd is only an indication that the animals were exposed to the virus at sometime during their lives. Animals which become infected after about six months of age normally develop SN antibody by 14 days post infection and clear the virus (Brownlie *et al.*, 1987; Harkness *et al.*, 1987). Once immune, the animal appears to have life long resistance to disease caused by BVDV, but may show serological evidence of subsequent reinfection (Brownlie *et al.*, 1987). In our present study, rises in antibody titre were observed in five heifers between 12 and 24 months of age. This may have been due to collecting the first sample before the peak antibody titre had been reached or may be evidence of reinfection occurring during the 12 month period. For example heifer number seven had the same antibody titre on two occasions separated by four weeks. However on a third occasion, some eight months later, there was a definite rise in titre suggesting reinfection had occurred in the intervening period. Previous workers have shown that peak antibody titres are reached by ten weeks after initial infection and remain at the same level for about three years (Kahrs *et al.*, 1966).

When different age groups on this farm were compared there was a small increase in geometric mean titres with increasing age suggesting that periodic reinfection may have been occurring. However statistical analysis of these results indicated that the differences in titre were not significant at the five percent level.

Four animals in the milking herd were identified which did not have antibody to BVDV (titre <0.6) and one which had a very low titre (0.9). When these animals were retested almost 12 months later (results not shown) there was no change in titre. From our understanding of the biology of BVDV infections these animals are either persistently infected and immunotolerant or they have some how escaped exposure to infection throughout their lives. It is believed the second alternative is highly unlikely in this herd where , on serological evidence, infection has been so widespread. Such persistently infected animals would constitute a continuing source of virus in the herd. Other investigators have found that persistently infected animals make up 0.5-2% of the cattle population (Ames, 1986; Ernst *et al.*, 1983). The number of animals in a herd which are shedding virus as well as the animal density are probably both important in determining the rate of spread of infection within a herd. The attack rate for BVDV infection has been computed to be between 0.002 and 0.06 per susceptible animal per day for grazing cattle compared with 60% when susceptible cattle were yarded overnight with a persistently infected animal (Littlejohns, 1988).

In the cohort study it was found that the antibody titre of calves' sera, except for that of one calf, reflected the titres of their dams. The one calf which was antibody negative remained so throughout the 12 month duration of the study and had probably failed to ingest colostrum from its dam during the first 24 hours of life. This theory is substantiated by two findings. First, as described in the next chapter, no virus was isolated from this animal. Secondly none of the calves with which it was running seroconverted as would have been expected if it was a persistently infected, immunotolerant animal. The fact that the antibody titres of all of the remaining calves declined steadily over the following months indicates that the antibodies detected in their sera were maternally derived and not the result of *in utero* infection. Prior studies have demonstrated that calves can acquire antibodies to BVDV from colostrum during the first 24 hours of life (Brambell, 1970; Butler, 1973). The estimate of a half life of approximately 25 days for maternally derived anti-BVDV antibodies is similar to the estimates of Menanteau-Horta *et al.*, (1985) for anti-BVDV antibodies and Douglas *et al.*,(1989) for the decline in maternally derived IgG in cattle.

The antibody titres of the calves had declined to < 0.6 by three to eight months of age depending on their initial titre. This compares with other

estimates of eight and a half and nine and a half months (Kendrick and Franti, 1974) and four to 12 months (Coria and McClurkin, 1978). Antibody responses to BVDV were not detected in any of the calves up to 12 months of age. This may have been related to the management system on this farm which led to autumn born calves having little to no opportunity for close contact with older animals which may have been a source of infection.

The prevalence of titres in the bobby calves (62%) was significantly lower than that of the cohort of calves on the farm (95%) at the same age. Many of the bobby calves had low titres or were negative (titres < 0.6). This may indicate that many of these calves had insufficient intake of colostrum or that the dairy farm which we studied was not typical of other farms in the region with respect to the prevalence of BVDV infection. Gammaglobulin levels were low in all of the bobby calf sera but there was no correlation between titre of anti-BVDV antibodies and level of total gammaglobulins (data not shown).

A serological investigation alone is not always sufficient to determine the current status of an animal or group of animals with respect to BVDV infection. Presence of SN antibodies indicates that the animal was exposed to virus at some time in the past when it was immunocompetent or that the antibodies were maternally derived. Isolation of virus is usually required to determine the current status of infection and a second sample four weeks later may be necessary to differentiate transient from persistent infection. Such information would be required before a specific control strategy was planned.

In the herd in this present study the cohort group was still antibody negative at the final sampling at 12 months of age. Unless they are exposed to BVDV and become immune during the subsequent 3 months they will enter pregnancy with a fully susceptible status. Contact with persistently infected and shedding animals at critical stages in pregnancy could have severe consequences (Baker, 1987; Duffel and Harkness, 1985).

SUMMARY.

i) Ninety seven per cent of the animals tested in the herd and 62% of the bobby calves from the abattoir had SN antibody. The geometric mean titres of different age groups showed a slight increase with age from one to five years of age and then declined in older animals.

ii) There was no statistically significant difference in SN antibody distribution in milking cows (97%), replacement heifers (100%) and one to four day old calves (95%) at five percent level.

iii) The prevalence of SN antibody in the sampled bobby calves from the abattoir (62%) was significantly lower ($p < 0.001$) than the animals tested on the specific dairy farm (95%, 97% and 100%).

iv) Maternally derived antibody in one to four days old calves, at titres similar to that of their dams, indicated that efficient transfer of antibodies from cows to their calves occurred. Levels declined steadily and SN antibody was undetectable by the age of eight months. The half-life of the maternally derived antibody was calculated from the regression line ($y = 2.5711 + (- 0.3741)x$) to be 25 days.

v) The results of repeated sample collection and testing from replacement heifers supported the hypothesis that reinfection with BVDV of animals with lower antibody titres occurs and results in increasing antibody titres.

CHAPTER FIVE

Virological studies on the epidemiology of infection with BVDV in a dairy herd.

INTRODUCTION.

The disease caused by infection with bovine viral diarrhoea virus (BVDV) was first recognized in association with epidemics of acute and often fatal disease characterized by diarrhoea and erosive lesions of the digestive tract (Olafson *et al.*, 1946). The aetiological connection between BVDV and the recognized disease syndromes was established when a cytopathic virus from a calf that had died from virus diarrhoea was isolated in cell culture (Gillespie *et al.*, 1960). Since then several strains of cytopathic and non-cytopathic biotypes of BVDV, most of which were antigenically closely related, have been isolated (Coria *et al.*, 1984; Heuschele, 1975; Roberts *et al.*, 1988).

While the presence of cytopathic biotypes is readily recognized in cell culture by the cellular degeneration which they cause, the presence of noncytopathic strains is less readily detectable. Viral interference assay (VIA) (Gillespie *et al.*, 1962), antigen detection by fluorescent antibody tests (FAT) (Fernelius, 1964) or immunoperoxidase staining (Hyera *et al.*, 1987) have been successfully used to detect noncytopathic biotypes of BVDV. Both cytopathic and noncytopathic biotypes are capable of inducing disease in infected animals (Straver *et al.*, 1983).

In animals which are infected with BVDV, viraemia may be either transient or persistent. Transient viraemia results from a primary postnatal infection and lasts for only about two weeks (Brownlie *et al.*, 1987). When antibody is produced, it persists for life and is thought to protect the animal from reinfection with BVDV (Kahrs,1966), however there is evidence that reinfection occurs in some animals which show elevation of previously stable or declining antibody titres (Chapter four; Roeder and Harkness, 1986).

Intrauterine infection at appropriate stages in gestation may result in immunotolerance and a viraemia which persists throughout postnatal life.

Persistently infected animals, although few in number, maintain the virus in a population. Approximately one to three percent of the animals in a herd may be identified as being persistently infected (Ernst *et al.*, 1983). Infection spreads rapidly to susceptible cattle which are in close contact with persistently infected animals. This is in contrast to the inefficient transmission from transiently infected cattle, and reflects the higher titre of virus in secretions, and the duration of viraemia and shedding by persistently infected animals (Roeder and Harkness, 1986). BVDV was isolated repeatedly over more than three years from blood and several secretions of a bull, which was persistently infected (Coria and McClurkin, 1978).

This present study concerns the isolation of BVDV from cattle in the dairy herd which was previously studied serologically (described in chapter four) and emphasises the detection of persistently infected animals and their possible role in the maintenance of infection within the herd. The sensitivity and specificity of the methods used for virus isolation have also been evaluated.

Materials and methods

Collection of samples. The dairy farm under investigation and the methods for collection of samples have been described in chapters two and four. Blood clots were stored at -70^{0}C until they were processed for virus isolation.

Cell culture. Bovine foetal lung (BFL) primary cell cultures were established as described by Goldsmit and Barzilai (1975).

Preparation of primary BFL cell cultures was performed by tissue culture procedures described in chapter two. The cells were tested for adventitious BVDV by viral interference assay and immunofluorescent antibody tests at the fourth passage level. Batches of cells which gave negative results in the above tests were subpassaged and were used at the seventh to tenth passage level for inoculation of blood clots in attempts to isolate BVDV.

Results

Cytopathic BVDV was not detected in any sample. Twenty one samples (6.1%) were positive when tested by VIA. When the positive samples were tested by FAT 15 samples were found to be positive in both tests.

Sixteen of the 21 animals from which virus was isolated at the initial sampling were sampled a second time after 12 months. Virus was isolated from the second sample collected from five of these animals. The results of virus isolation and the serological status of the 21 animals is shown in (Table 5-1).

The SN antibody titres in the first test ranged from < 0.6 to 2.7 (log10). Animals which were viraemic at this sampling had titres ranging from < 0.6 to 2.7 while, of the five that were still viraemic at the second sampling, the titres ranged from < 0.6 to 1.8. One animal which had a titre of < 0.6 and was virus positive at the first sampling was not available for the second sampling. Thus of the 16 animals which were tested twice, 11 showed a rise in titre, and virus was not isolated from them at the second sampling.

The age of viraemic animals varied from one to more than eight years. However most animals were between two and five years old. One animal, from which virus was isolated and had a titre of < 0.6, was eight years old (Table 5-1).

The " comparative sensitivity and specificity" of VIA and FAT were evaluated. The 26 samples which were positive in the VIA and a further 67 samples which gave negative results were tested by FAT (Table 5-2).

Traditionally the sensitivity and specificity of a test is obtained by comparing the results of the test with a more definite test (benchmark). However in the present study a slightly different approach was taken. Although the FAT could have been considered the " benchmark " on which to calculate the sensitivity and specificity of the VIA, it was decided to use both as " benchmarks ".

The VIA had a sensitivity of 86.9%, a specificity of 91.4% and a predictive value of 76.9% when compared with the FAT. The FAT had a sensitivity of 76.9%, a specificity of 95.5% and a predictive value of 86.9% when compared with the VIA. When the results of both tests on the total 93 samples were compared there was 88.4% agreement for positive samples and 95.7% agreement for negative samples.

TABLE 5-1.

Detection of virus and neutralizing antibody in blood of cattle.

		First testing			Second testing		
No.of animals	Age of animals (years)	VIA	FAT	SN	VIA	FAT	SN
1	4	+	+	< 0.6	+	+	< 0.6
2	4	+	+	< 0.6	+	+	< 0.6
3	2	+	+	< 0.6	+	+	< 0.6
4	8	+	+	< 0.6	NT*	NT	NT
5	3	+	+	0.9	+	+	0.9
6	3	+	+	1.8	+	+	1.8
7	2	+	+	0.9	-	-	2.7
8	1	+	+	0.9	-	-	3.0
9	10	+	+	1.8	NT	NT	NT
10	3	+	+	1.8	-	-	2.4
11	3	+	-	1.8	NT	NT	NT
12	3	+	+	1.8	-	-	2.7
13	2	+	+	2.1	-	-	3.0
14	5	+	+	2.1	-	-	2.4
15	5	+	+	2.1	-	-	3.0
16	11	+	+	2.1	-	-	3.0
17	9	+	-	2.4	-	-	2.4
18	4	+	-	2.4	NT	NT	NT
19	3	+	-	2.4	-	-	2.7
20	5	+	-	2.4	NT	NT	NT
21	8	+	-	2.7	-	-	3.0

Samples were collected for the second test at 12 months after the first test.

VIA = viral interference assay

FAT = fluorescent antibody test

SN = serum neutralizing antibody titre (log10)

* NT = not tested. (these animals were not available for retesting).

TABLE 5-2.

Comparison of the viral interference assay and fluorescent antibody test in detecting viraemia.

	Fluorescent antibody test		
Viral interference assay	positive	negative	total
positive	20	6	26
negative	3	64	67
total	23	70	93

DISCUSSION.

The results of the serological and virological studies demonstrated that BVDV is endemic in this dairy farm. All of the viral isolates which were obtained from cattle on this farm were of the noncytopathic biotype. When 16 animals were sampled a second time at 12 months after the initial sampling, virus was not reisolated from 11 of them. Of these 11 animals 10 had rising antibody titres but the antibody titre of one animal remained the same. It is concluded that these animals were only transiently infected. Viraemia in transiently infected animals persists for about 15 days (Brownlie *et al.*, 1987; Duffel and Harkness, 1985). An important question that is raised by these results is whether the initial sampling of these animals coincided with the first time that they had been infected or whether it coincided with a time of reinfection which occurred in the face of relatively low levels of preexisting antibody. The idea is favoured that reinfection is occurring. It is believed to be highly unlikely that animals aged eight to 11 years in this endemically infected herd (e.g., animals No. 4,9,16,17,21) would only be infected with the virus for the first time at our initial sampling. It seems more likely that reinfection can occur if serum antibody levels decline sufficiently and there is exposure to a high titre of virus such as would be shed by a persistently infected animal. On the basis of these results it appears that serum antibody titres less than 2.4 may not always protect against reinfection. Other workers have suggested that titres ranging from 0.9 to 2.1 or even higher may be required for protection (Howard *et al.*, 1989).

The animals from which virus was isolated were not showing any clinical signs of infection with BVDV at the time of sampling. This was also true of the five animals from which virus was isolated on both occasions. It was presumed that these five animals were persistently infected. In three of these animals SN antibody was not detected on either occasion and these animals are considered to be specifically immunotolerant and persistently infected. The other two animals which had low, but constant, titres of SN antibody were persistently infected yet not fully immunotolerant. Persistent infection with low SN antibody titres has been previously described and may be related to strain or serotype specificity of the immune response (Bolin *et al.*, 1985). Many persistently infected animals die early in life, however some may appear healthy and live for many years transmitting the infection to animals which are in contact and also vertically to their offspring (Corapi *et al.*, 1988). In this context it is important to note that two of the immunotolerant, persistently infected animals in this study (No.1,2) were four years old and a third animal (No.4), which may also qualify, was eight years old.

When animals in a herd are exposed to BVDV for the first time the infection spreads quickly and the infected animals develop high titres of SN antibody and become immune (Brownlie *et al.*, 1987). The virus may be eliminated from such a herd if all of the animals become immune, but the second generation will be fully susceptible when maternally derived antibody has waned (Brownlie *et al.*, 1987). The introduction of persistently infected bulls or heifers into this herd will efficiently spread the virus to fully susceptible animals. If these animals are in early pregnancy it may result in foetal infection and all the sequelae of foetal infection may be manifested depending on the period of gestation. In the dairy farm where the present study was conducted this does not seem to be the case. The bulls introduced to the herd for mating had titres of SN antibody to BVDV and were not viraemic. Also the level of herd immunity was high although some age groups, such as the cohort of calves at eight to 12 months of age, were susceptible to infection.

In some farms, replacement heifers are reared in isolation from adult animals until they reach breeding age. If these heifers are exposed to BVDV during early pregnancy, foetal infection with various sequellae may result. Persistent infection may be one of these manifestations and infection may thus be maintained in the herd from generation to generation (Brownlie *et al.*, 1987; Littlejohns, 1988). In the farm, where the current study was conducted, persistently infected animals, possibly as old as eight years, were identified. Since such animals must have been infected *in utero*, BVDV has been present on this farm for many years.

As stated in chapter two, there are two seasonal calving periods (autumn and spring) in this dairy farm. During our time of study the autumn calves born in 1988 were rotated through the paddocks separately and isolated from other animals. These calves became susceptible when maternally derived antibody waned by three to eight months of age. They remained antibody negative until 12 months of age, the limit of the period of study. On the other hand, calves born in the spring of 1987 were tested at the age of one year. All of them had antibody, and virus was not detected except in one heifer which had a rising antibody titre but no detectable viraemia when retested at the age of two years. In contrast to the calves born in autumn, these spring calves had been mixed with the adult herd in small groups in the same paddocks throughout the first 12 months of life. This allowed contact with persistently infected animals in the adult herd.

In this herd 5/340 (1.4%) of the animals which were tested were persistently infected. One would expect that some cohorts of calves would contain at least one calf that was immunotolerant and persistently infected. This was not the case in the cohort which was studied. The calves in a cohort containing a persistently infected calf would be expected to become infected as their titres of maternally derived antibody decline. Mild to severe clinical disease may be seen in calves aged six to 12 months, which would be transiently infected and thereafter immune (Potgieter, 1988).

This study indicated that the pattern infection with BVDV in this herd varied from year to year. The presence of a persistently infected calf within the cohort of calves or early exposure of calves to adult animals in a group where persistently infected animals were present would affect the pattern. The chance of exposure of animals to BVDV therefore depends largely on management. Appropriate management strategies, developed with a knowledge of the epidemiology of BVDV, could minimize the effects of infection. For example, deliberate exposure of heifers to BVDV before they reach breeding age has been suggested as a method of avoiding losses due to *in utero* infection (Littlejohns, 1988).

Both VIA and FAT are useful tests for detecting virus in tissue culture and close agreement (88.4%) between these tests was found in the present study. VIA is more convenient when working with large numbers of samples in tissue culture plates. It also appeared to be more sensitive, but less specific than FAT in this study. It does require, however, the availability of a challenge virus and suffers from ill-defined endpoints for quantitative assays (Roberts *et al.*, 1988). The interpretation of the VIA was less subjective than with the FAT however the FAT was still a useful and reliable test.

Infection with BVDV is endemic in the dairy herd which was investigated. Of the animals which were tested, 1.4% were found to be persistently infected, and these would provide an adequate mechanism for maintenance of the virus. An alternative strategy to the deliberate exposure of animals before breeding age, would be the isolation and culling of the persistently infected cattle from the herd. To achieve this option a laboratory test with high specificity and sensitivity would be required for identification of those animals. In the absence of a reliable vaccination programme, this approach would have the disadvantage of producing, in due course, a fully

susceptible herd and the consequences of reintroduction of virus from outside could be disastrous.

Summary.

i) BVDV is endemic in the dairy herd and both transiently and persistently infected animals were detected. Of 340 animals tested, 21 were viraemic on at least one occasion and five of these were persistently infected.

ii) In a proportion of persistently infected animals SN antibody was not detected (< 0.6), but two such animals had low titres of SN antibodies (0.9 and 1.8). The age of infected animals ranged from two to more than eight years. There is evidence that animals with low titres were reinfected with BVDV, presumably from persistently infected animals, which provided an important mechanism for maintenance of the infection on the farm.

iii) The VIA and the FAT were compared for sensitivity and specificity and both tests were found to be in close agreement (88.4%). The VIA was more sensitive, but less specific than the FAT. It was also more convenient when working with large numbers of samples in multiwell tissue culture plates.

iv) A laboratory test with high specificity and sensitivity is necessary if it is desired to detect the low prevalence of persistent infection with BVDV in the cattle population in New Zealand.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION.

BVDV is an important pathogen which causes a wide range of clinical syndromes in cattle, other wild and domestic ruminants, and pigs. The sources of BVDV in a herd are either transiently or persistently infected animals which shed the virus into the environment.

Transmission may be direct or indirect, and inhalation or ingestion of infected saliva, oculonasal discharge, uterine discharges, urine and faeces are principal means of infection. Transmission also may occur through infected semen, and transplacentally.

Transplacental infection of the foetus is well recognized and is epidemiologically significant. The sequelae of foetal infection vary depending on the stage of gestation of the pregnant animal. Persistent infection with immunotolerance is one of the results of intrauterine infection. Persistently infected animals do not have SN antibodies or, rarely, may have low titres. Some of the persistently infected animals are stunted and unthrifty, however a proportion of them remain apparently healthy for several years and may live to maturity or breeding age. They constitute an important source of BVDV for other animals in the herd, give rise to persistently infected offspring, and also they may develop fatal mucosal disease.

Most infections in susceptible adult cattle are subclinical. The transiently infected animals develop SN antibody within about two weeks and are protected for their life time. After primary infection, peak titres are reached by ten weeks and remain high for about three years. However, recent studies on the epidemiology of infection of cattle with BVDV (Brownlie *et al.*, 1987) and the results of the present study demonstrate the possibility of reinfection of animals with low antibody titres.

The presence of SN antibodies to BVDV in a cattle population indicates that the animals have been exposed to the virus at sometime during their life time or that they have acquired antibody from their dams, which had

been infected with BVDV. Surveys conducted 18 years ago to estimate the prevalence of SN antibody to BVDV in the New Zealand cattle population found antibody in 41 to 66 per cent of animals examined. Sporadic cases of mucosal disease and the appearance of calves with a range of congenital defects attributable to infection with BVDV have been reported in later years.

Epidemiological aspects of infection with BVDV in a commercial dairy farm were studied by measuring titres of SN antibody and attempting to isolate virus from animals in several age groups. The mechanisms of maintenance of the infection in a relatively confined dairy farm and the distribution of SN antibodies in various age groups have been studied in detail. The duration and half-life of maternal antibody in calves was also determined.

SN antibodies to BVDV were detected in the sera of 97.1% of the adult milking herd and replacement heifers. The geometric mean titres of the different age groups showed a slight increase from one to seven years old but subsequently decreased. Statistical evaluation of this data however indicated that the differences were not significant at the five percent level. The SN antibody titres of a cohort of 21 calves that were first sampled at one to four days of age were proportional to those of their dams and declined steadily, with a half-life of 25 days, so that by eight months of age all calves tested were negative. However, one calf remained antibody negative from birth to 12 months of age and had probably failed to ingest colostrum. Initially it was postulated that this calf was persistently infected. However, no virus was isolated from this calf and other calves which were in close contact with it did not seroconvert after their maternally derived antibodies waned.

SN antibody titres in a group of bobby calves which were obtained from a local abattoir were significantly lower than those of the cohort of calves on the farm. Presumably many of these calves had not acquired sufficient colostrum or their mothers had never been exposed to BVDV. If this latter explanation is correct then it suggests that the prevalence of infection with BVDV may vary greatly from farm to farm.

The small increase in geometric mean titres with increasing age suggested that periodic reinfection may have been occurring. This hypothesis is supported by the virus isolation studies in which some animals, from one to more than eight years old were tested twice within 12 months. Persistently infected animals were identified in the herd, and animals with lower antibody

titres appeared to be susceptible to reinfection which resulted in raised antibody titres. A SN antibody titre of 2.1 does not seem to protect the animals against reinfection. In fact virus was isolated from one animal with a SN antibody titre of 2.7. Consistent with this finding are earlier studies which demonstrated that SN antibody titres between 0.9 and 2.1 did not protect calves against experimental infection (Howard *et al.*, 1989). It is possible that animals with low titres can be reinfected if the titre of challenge virus is sufficiently high.

In the natural history of BVDV a major mechanism for maintenance of the virus in the population is persistent infection. Any other way in which virus is maintained in the ecosystem may be subclinical (inapparent) infection although such animals shed virus for a shorter time and at lower titres. Thus, when BVDV is introduced to a virgin area it spreads very swiftly within a herd, often with little recognizable clinical effect.

The other important factor in the survival and spread of BVDV in the herd is the availability of a susceptible population. The availability of both a continuous source of BVDV and a susceptible population is required. The particular farm management system is important in determining when the susceptible population, young animals whose maternal antibody titres have declined, comes in contact with a source of virus,usually persistently infected animals in the adult herd. Since the long term survival of BVDV requires that it should continue to be transmitted from one host to another, the virus is expected to disappear from the population if its potential supply of susceptible hosts is exhausted. This usually happens on farms, where there are no persistently infected animals in the herd.

On commercial dairy farms in New Zealand the animals are grazed on pasture throughout the year. Opportunities for reinfection with BVDV in such a herd would be lower than in a herd in a colder climatic zone, where the animals are held inside for a proportion of the year. This leads to a high concentration of animals in a small area, enables close contact and may predispose the animals to stress and other debilitating factors. Infection with BVDV is significant in feedlots because it causes immunosuppression and increases the severity of secondary respiratory disease. Concurrent infections with BVDV and parainfluenza type 3 viruses and *P.haemolytica* play an important role in the pathogenesis of shipping fever. Transport and the high concentration of animals in feedlots are considered as debilitating factors,

which may exacerbate the immunosuppression which results from infection with BVDV.

Although the distribution of SN antibodies within herds varies in different parts of the world, the proportion of persistently infected animals remains low and does not exceed four per cent of the herd.

The host range of the virus is also significant in determining the survival of the virus in nature. Domestic and wild ruminants, and pigs are known to be infected with BVDV under natural conditions. Cross infection of the virus between cattle and sheep has been reported. Persistent infection and immunotolerance, if it occurred in these host species after natural infection, would have epidemiological significance.

The presence of different strains of BVDV would also have an impact on the survival of the virus in a population. Mutation from the noncytopathic biotype has been suggested as a mechanism for the generation of cytopathic biotype. The cytopathic biotype is usually associated with fatal MD, which occurs in animals which have a persistent infection with a noncytopathic biotype of BVDV. Several strains of the cytopathic and noncytopathic biotypes have been isolated from animals infected with BVDV. In the present study a strain of BVDV isolated in New Zealand and designated BVD-MDBK, was effectively neutralized by anti-serum raised against the NADL strain of BVDV. The NADL strain was used in this study to determine the prevalence of SN antibodies in bovine sera in New Zealand in the belief that it was antigenically related to local strains of BVDV. The results of the serological surveys and serological comparisons of local strains vindicate this assumption.

The SN test is a highly specific test and a titre of 0.6 or greater has been regarded as indicating either a previous encounter with BVDV or passive immunity. In the present study the reproducibility of the SN test was monitored by using a standard reference serum each time the test was run. The results fluctuated only one dilution either to the left or right side of the mean value.

Generally a serological test, such as the SN test or ELISA, is an important tool to evaluate eradication and immunization programmes and also to assess the past history of a group of animals. By detecting SN antibodies to BVDV in various age groups of the herd, it is possible to determine the degree of endemicity of the infection, or how long it has been in the herd. Estimations

of prevalence or incidence can be made when pairs of serum samples are obtained several weeks apart from individual animals. Initial appearance of antibody in the second specimen or a rise in antibody titre gives an indication of the time that infection occurred. The other point in serological studies of BVDV to be considered, is the presence of maternally derived antibody and its duration. In the present study the duration of maternal antibody in the cohort of calves varied from three to eight months. In other works it has also been detected up to the age of 12 months.

A serological test alone may not be adequate to determine the current status of cattle with respect to infection with BVDV. The antibody negative animals may be either recently infected, persistently infected or fully susceptible to BVDV. Isolation of the virus and repeated serological testing is required to differentiate these different states of infection. While many of the persistently infected animals may be antibody negative, a few animals may still have low antibody titres, which may be stable over a long period of time. In the current study two of the persistently infected animals had low titres of 0.9 and 1.8, which remained unchanged when retested 12 months later.

Serological tests, other than the SN test, have been used for the detection of antibody to BVDV. The agar gel diffusion precipitation test is considered to be less sensitive and less specific. Provided the appropriate reagents and protocol are available the ELISA is considered as sensitive and specific as, but not higher than the SN test. The advantage of the ELISA over the SN test is that once it has been standardized, it is easier and cheaper to run, and the results are attained within hours, while several days are required to read the results of the SN test. Low reproducibility and nonspecific reactions have been major impediments to attempts to establish an ELISA for antibody detection by the present author (data not shown).

Isolation of cytopathic or noncytopathic virus from clinically affected animals, or from animals which are apparently healthy is of diagnostic significance. It is generally accepted that, for those viruses which can be isolated in cell culture, virus isolation is a benchmark against which other antigen detection methods are evaluated for sensitivity and specificity. However, factors which are likely to affect sensitivity of virus isolation procedures are the initial titre of virus in the sample, the procedure of sample collection, transportation and the availability of an adequate cold chain, processing procedures in the laboratory, the sensitivity of the cell culture system used for inoculation and aseptic handling.

When the comparative sensitivity and specificity of virus isolation techniques used in the current study were evaluated, the viral interference assay appeared to be more sensitive but less specific than the fluorescent antibody test. The two tests had an 88% agreement so both tests are approximately equal in their reliability for routine virus isolation. The viral interference assay is easier to conduct if large numbers of samples are to be processed and multiwell tissue culture plates can be used.

Unfortunately, no specific, reliable and practical control measures can be recommended as a result of this study. Obviously much useful information was obtained on the epidemiological aspects of the infection including, the prevalence of SN antibody in the herd, the duration and half-life of maternal antibody, the possibility of reinfection of animals with lower titres, identification of persistently infected animals, and evaluation of methods used for virus isolation. An understanding has been obtained of when animals within the herd are most likely to be first infected with BVDV and the role of management factors in determining this time. An evaluation of the sensitivity and specificity of the virus isolation methods has also been made so it is possible to choose one of the two methods depending on the number of samples to be processed.

A concept of maintaining a herd which is free of BVDV for a long time may not be practical, because of the various routes by which the virus may enter the farm. Vaccination of calves from six to ten months of age with modified live or inactivated vaccines has been recommended by some workers. However, the fear of immunosuppression, exacerbation of secondary respiratory disease and sometimes of fatal MD in calves are limitations to the use of live vaccines. Short term protection and high cost have limited the use of inactivated vaccines. A screening test for the identification and elimination of persistently infected animals has been suggested. Because of the low prevalence of persistent infection, any screening test would have to have high sensitivity and specificity and be technically feasible.

In the short term, deliberate inactivity may appear to be effective in controlling the infection, however, it is not predictable. While most infections will be subclinical and lead to long term immunity, there will be a small number of persistently infected animals which may lead to infection of young stock at times when the consequences may be disastrous.

If eradication of BVDV from a farm is not feasible, then the losses related to this infection may be minimized by appropriate management systems. It is important for the replacement heifers to have high titres of antibody before the breeding season. This may be achieved by exposure of these heifers to persistently infected animals under natural conditions or by vaccination with inactivated vaccines at the age of 12 months.

Despite the better understanding of the nidality of BVDV in the natural ecosystem, and the availability of highly sensitive and specific tests in diagnostic virology, infection with BVDV is likely to persist in the cattle population in New Zealand for many years to come.The estimated cost associated with the infection is lower than the cost of contemplated specific control strategies; the virus is ubiquitous and interspecies transmission and persistent infection in a wide range of host species is more complicated than previously assumed. There are some special circumstances where control or eradication of BVDV is demanded so mandatory requirements have been put in place at both the national and international level. Such examples include the use of bulls in AI centres and the export of livestock to some countries, also, in countries, where SFV and BVDV could complicate laboratory diagnosis of infectious diseases, especially in the pig industries, eradication of BVDV would be highly desirable.

Because of the presence of other devastating animal and zoonotic diseases in the developing countries, such as Ethiopia, the control of BVDV is not likely to be a priority, however, the epidemiology of this disease could be used as a model for assessment of other prevailing diseases in those countries.

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Appendix I

Buffer and solutions used for cell culture

1. Antibiotic-Trypsin-Versene (ATV)

0.5 g Trypsin
0.2 g Versene (EDTA sequestric acid)
8.0 g NaCl
0.4 g KCl
1.0 g Dextrose
0.58 g NaHCO3
$2x10^5$ IU Penicillin
100 mg Streptomycin
0.02 g Phenol red

Made up to 1 L with distilled water. Sterilized by filtration. Stored at -4^oC.

2. Eagles minimum essential medium (MEM)

10.0 g MEM powder
1.0 g NaHCO3

Made up to 1 L with distilled water. Sterilized by filtration. Stored at -4^oC. pH 7.2-7.4

3. Penicillin, Streptomycin, Kanamycin (PSK)

10.0 g Streptomycin
10.0 g 1-mega vial Penicillin
10.0 g Kanamycin

Made up to 1 L PBS. Sterilized by filtration.

4. Phosphate buffered Saline (PBS)

8.0 g NaCl
0.2 g KCl
1.15 g Na2HPO4
0.2 g KH2PO4

Made up to 1 L with distilled water. Sterilized by autoclaving 15 lbs for 15 minutes. pH 7.2-7.4

5. Trypsin solution

0.40 g trypsin (1:250) powder
100.0 ml PBS. Stored at -4^{o}C.

6. Trypan blue

0.2 g Trypan blue powder
100.0 ml PBS

Dispensed in 1.8 ml aliquots and stored at -4^{o}C

Appendix II

I. Papers ready for publication from this thesis

1. Abraham, G., Wilks, C.R., Blackmore, D.K. (1989): Epidemiology of BVDV infection in a dairy herd. I. Serological studies. N.Z. Vet. J.
2. Abraham, G., Wilks, C.R., Blackmore, D.K. (1989): Epidemiology of BVDV infection in a dairy herd. II. Virological studies. N. Z. Vet. J.

II. Paper published from related work to this thesis.

1. Wilks, C.R., Abraham, G., Blackmore, D.K. (1989): Bovine pestivirus and human infection. Lancet 1: 107-108.
2. Wilks, C.R., Abraham, G., Blackmore, D.K. (1989): Does BVD virus infect man ? N.Z. Vet. J. 37: 86-87.