Mastitis in New Zealand Dairy Herds:

I. Management, diagnosis, and treatment of subclinical mastitis

II. Phenotypic and genotypic characterisation of Streptococcus uberis isolates

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Abstract

Four scientific trials were undertaken to extend the scientific research on mastitis in New Zealand dairy herds, producing milk seasonally from grazed pastures.

Trial 1:

Aim. To compare the effect of drying-off techniques on the prevalence of new intramammary infections.

Conclusions. A 48-hour intermittent drying-off technique increased the quarter prevalence of mastitis caused by major and minor pathogens or major pathogens alone, at dry-off. There was no significant difference in the prevalence of mastitis in quarters dried-off with a 24-hour or 48-hour intermittent milking technique, during the first 12 days of the subsequent lactation. This may have resulted from higher culling (due to apparently unrelated causes) of infected cows from the 48 hour group.

Trial 2:

Aims. To assess the economic value of treating subclinical mastitis based on electrical conductance and/or rapid mastitis test results and to determine the cure rates and economic outcome to the dairy farmer.

Conclusions. The Rapid Mastitis Test or electrical conductance diagnosed 66% of the quarters infected by major pathogens. Cure rates were 58% in infected untreated quarters and 61% in infected quarters treated with a course of intramammary antibiotics. An assessment of costs and benefits from treating infected quarters, plus the inevitable cost of treatment of uninfected quarters, resulted in an overall net loss of approximately $9.18 per quarter treated.

Trial 3:

Aims. To determine the antibiotic sensitivity patterns of 150 and 180 *Streptococcus uberis* isolates cultured from subclinical and clinical cases of mastitis respectively, in New Zealand dairy cattle from 15 different regions, using a disk diffusion assay. To assess the suitability of antibiograms for subtyping of *Streptococcus uberis* isolates for epidemiological studies.

Conclusions. The 330 isolates fit into 17 different antibiogram patterns. Ninety-five percent of the isolates followed one of five common antibiogram patterns. Eighty percent of the isolates were of

antibiogram pattern C. The only significant difference between the subclinical and clinical *Streptococcus uberis* isolates tested in this study was the clinical isolates were more sensitive to cloxacillin. Similarities in antibiograms between the study isolates and the isolates from the United States (McDonald *et al.*, 1976) were noted for cloxacillin, cephalothin, erythromycin, lincomycin, penicillin G, and streptomycin. The relatively low number of antibiogram patterns resolved from the 330 *Streptococcus uberis*, limits the usefulness of the technique as a solitary tool for epidemiological studies in New Zealand dairy herds.

Trial 4:

Aims. To determine and compare the restriction endonuclease fragment pattern of *Streptococcus uberis* isolates from subclinical and clinical cases of mastitis in New Zealand dairy cows, from 15 different farming regions in New Zealand. To compare the pulsed-field genotyping technique with antibiogram typing of *Streptococcus uberis* isolates within 8 different farms.

Conclusions. The 343 *Streptococcus uberis* isolates exhibited 330 different restriction endonuclease fragment patterns, indicating at least several hundred genetically different strains of *Streptococcus uberis* isolates in New Zealand capable of causing mastitis in dairy cattle. Genetically different and similar strains were identified within the same quarter of an individual cow, different quarters from the same cow, different cows within the same farm, and from different cows from the same or different districts, farming regions or islands. The high degree of dissimilarity among the isolates tested is an indication that *Streptococcus uberis* infections in New Zealand dairy cattle are largely due to the opportunistic nature of the organism in the cow's environment. Prevention and treatment of *Streptococcus uberis* mastitis will therefore need to be directed at a multitude of different strains present throughout the country as well as in individual herds.

Fewer antibiogram patterns were defined on each individual farm compared to the number of pulsed-field gel electrophoresis patterns on those farms. The pulsed-field gel electrophoresis typing technique appears to be a more discriminatory test for typing *Streptococcus uberis* isolates on 8 New Zealand dairy herds than antibiograms.

The information gained from these trials provides scientific research to strengthen the recommendations made in the Seasonal Approach to Managing Mastitis (SAMM) Plan, a mastitis prevention programme designed for seasonal dairy farmers in New Zealand.

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I am sure there is more, but let's get on with the thesis...

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Chapter 1

Introduction

Where there are cows there is mastitis

Mastitis is the inflammation of the mammary gland. This is the most costly disease affecting all aspects of the dairy industry from the cow to the consumer (Sischo *et al.*, 1990; Gardner *et al.*, 1990; Miller and Dorn, 1990; Bramley 1992). Ratafia (1987) stated that World-wide annual losses to mastitis equate to nearly 35 billion USD ($72 billion NZD). In Australia, mastitis cost the country $120 million AUD ($144 million NZD) per year. In the United States mastitis losses cost the country approximately 5.4 billion ($11 billion NZD) annually (Ratafia, 1987). Holdaway (1992) estimated the average annual cost of mastitis in a New Zealand dairy herd to be approximately $14,639, equivalent to approximately $86 NZ/cow in a herd of 170 milking cows, with a bulk milk somatic cell count (BMSCC) of 400,000 cells/ml. As in other countries, subclinical mastitis is the major cause of monetary losses in New Zealand dairy herds and US dairy herds (Blosser 1979). In New Zealand, approximately 62% ($9,000) of the monetary loss was due to the loss of milk production. Culled cows accounted for 32% of the total monetary loss, and the cost of therapeutics for treating clinical mastitis during lactation and dry cow therapy (DCT) was approximately 6% of the total (Holdaway, 1992). An additional labour cost for dealing with clinical cases and the cost of the discarded antibiotic contaminated milk were less than 1% of the total (Holdaway, 1992).

Historical trends

During the 1989/90 lactation season, 2.3 million dairy cows in New Zealand produced 330 and 242 million kilograms of milkfat and protein respectively (Anon, 1997). Eight seasons later, 3.2 million dairy cows produced 513 and 379 million kilograms of milkfat and protein respectively, in 1997/98 (Anon, 1998). The average herd size increased from 154 cows during the 1989/90 season to 220 in the 1997/98 season. New Zealand dairy cows are produce on average, approximately 585 L more milk than the cows of 20 years ago. Although there is a positive correlation between increasing milk production in cows and increasing prevalence of mastitis (Keefe and Leslie, 1997), the country's average BMSCC has been steadily declining. The national seasonal average BMSCC for 1989/90 was 358,000 cells/ml, but by the end of the 1997/98 season it had dropped to 195,000 cells/ml (Anon, 1998).

Changes throughout all levels of the dairy industry that have occurred may have accounted for the increase in the countries milk production and the lowering of the national mean BMSCC. The development of the Seasonal Approach to Managing Mastitis (SAMM) Plan, imposition of penalties for high BMSCC by dairy companies, increase use of therapeutics, more frequent bulk milk and individual cow somatic cell count testing, and the overall increase of farmer awareness and understanding of the disease are changes which have probably influenced the decreased average BMSCC.

Although the countries average BMSCC has declined, mastitis still occurs in New Zealand dairy herds. The SAMM Plan is a prevention programme designed to minimise and/or prevent the incidence of mastitis caused by *Streptococcus uberis* and

other potentially pathogenic organisms, in seasonal dairy herds. The recommendations in the SAMM Plan were initially based on the 5-point plan developed in England, with limited scientific research under New Zealand seasonal calving conditions.

The SAMM Plan is divided into 5 major periods for seasonal milk production (Table I). Within each period recommendations are made with regards to the ideal way to manage dairy cows during that period.

The present experiments investigated recommendations for the periods of drying-off, calving, and lactation. The first experiment examined the effect of two intermittent drying-off procedures on the incidence of subclinical and clinical mastitis at dry off and 12 days into the following lactation.

Table I: The main points to the 1994/95 SAMM Plan (Anon, 1995)

Late lactation period	⇒	*Review clinical cases* *Review cow counts* *Treat late season clinicals* *Decide dry cow antibiotic strategy*
Drying-off period	⇒	*Dry off abruptly* *Administer dry cow antibiotics* *Check quarters*
Dry period	⇒	*Test machine and correct faults* *Treat clinicals* *Enrol for somatic cell counting* *Set up recording system*
Calving period	⇒	*Use clean pasture* *Treat & record clinicals* *Teat spray* *Milk out completely* *Minimise suckling*
Lactation period	⇒	*Monitor machine & correct faults* *Treat clinicals* *Monitor bulk somatic count* *Practise good hygiene* *Use good milking technique* *Teat spray* *Manage teat condition*

The second experiment investigated the diagnosis, identification and treatment of subclinical mastitis during lactation. The identification of the causative agent(s) on a dairy farm is important to establish a prevention/treatment programme. Identification of the infected quarter(s) using "cow-side" diagnostic tests (Rapid Mastitis Test, individual somatic cell counts, and/or electrical conductivity meters) with subsequent treatment of clinical cases and particular subclinical cases is recommended in the SAMM Plan (Anon, 1995; Steffert, 1997).

Treatment of each mastitis case is recommended during all 5 periods of the SAMM Plan (Anon, 1995). It is not economical to do bacteriological cultures and sensitivities

on every subclinical and clinical case of mastitis in a herd. A standard antibiotic regime that could be used effectively against the common strains involved would alleviate the need for repetitive cultures and sensitivities. The third experiment examined the antibiotic sensitivity of *Streptococcus uberis* isolates, a common pathogen involved in subclinical and clinical cases of mastitis in New Zealand. This experiment examined the possible existence of a common antibiogram pattern among isolates from different farming regions in New Zealand. The presence of a common antibiogram pattern among New Zealand *Streptococcus uberis* isolates could be used to strengthen the economical management and treatment of *Streptococcus uberis* mastitis cases.

Prevention should be the first line of defence against mastitis, but establishing a treatment protocol for *Streptococcus uberis* mastitis is necessary when prevention measures are inadequate. *Streptococcus uberis* has been recognised as an important causative agent of mastitis in New Zealand dairy herds. Little is known about the epidemiology of *Streptococcus uberis* in New Zealand. The final experiment examines the genotypic characterisation of *Streptococcus uberis* isolates from multiple farming regions in New Zealand. The phenotypic (antibiogram testing) and genotypic (pulsed-field gel electrophoresis) typing techniques will be compared. Subtyping of *Streptococcus uberis* isolates provides information on possible sources of the organisms. A determination of genetic relationships among the isolates would provide important information for the widespread treatment and/or prevention of *Streptococcus uberis* mastitis.

The ultimate aims of all the experiments are to provide scientific evidence derived under New Zealand conditions that will strengthen the recommendations in the SAMM

Plan.

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Chapter 2

Literature Review

Mastitis in New Zealand:

New Zealand commercial dairy herds are kept on pasture throughout the entire lactation and dry period. Godkin (1997) suggests calving cows out on pasture, if there is a problem with intramammary infections (IMI) in a herd not normally pastured, because pastured animals have a lower prevalence of mastitis compared to cows housed indoors or in feedlots. Nevertheless, New Zealand continues to have problems with both contagious and environmental mastitis.

In New Zealand, Gram-positive bacteria are the most common aetiological agents involved in both subclinical and clinical mastitis cases (Brookbanks, 1966; McDougall, 1998). Roberts (1967) reported that 97% of all udder infections were caused by Gram-positive bacteria ((*Streptococcus agalactiae (S. agalactiae)*, *Staphylococcus aureus* (*S. aureus), Streptococcus uberis (S. uberis)*, and *Streptococcus dysgalactiae (S. dysgalactiae)*). Common agents involved in mastitis cases were *S. aureus* cultured from 54% of the cases and *S. uberis* from 32% of the cases (Holdaway, 1992). Collectively *S. agalactiae* and *S. dysgalactiae* constituted less than

12% of the cases. In a more recent survey, approximately 3820 milk samples were cultured at the 5 Ministry of Agriculture and Fisheries laboratories and the Alpha Scientific Laboratory during the 1995/96 season. Of these, 30% of cultures were positive for *S. aureus*, 25% for *S. uberis*, 15% for CNS, 8% for *S. dysgalactiae,* 2% for EC, 1% for *S. agalactiae*, and 2% of the samples cultured both *S. aureus* and *S. uberis*. The majority of these samples probably came from clinical cases and/or cows with elevated somatic cell counts (SCC) during lactation.

The number of mastitis cases caused by the contagious organisms *S. aureus* and *S. agalactiae* are declining in New Zealand (Brookbanks, 1966; Holdaway, 1992; McDougall, 1998). A decrease in contagious pathogens and an increase in environmental pathogens have been reported in other countries (Cullen, 1966; Bramley and Dodd, 1984; Oliver, 1988; Todhunter, 1995). This may be due to the increase use of post milking teat disinfectants, DCT and better milking hygiene practices (Godkin, 1997). Researcher workers have reported an increase in environmental streptococci (ES) isolations concurrent with declining contagious organisms (Oliver and Mitchell, 1984; Smith *et al.*, 1985; Oliver, 1988; Todhunter, 1995). *Streptococcus uberis* and *S. dysgalactiae* are the most prevalent of the environmental streptococci (Smith *et al.*, 1985; Oliver 1988; Todhunter 1995). *Streptococcus uberis* is one of the most frequently isolated major pathogens, which causes clinical mastitis in heifers in New Zealand and remains an important environmental pathogen involved in mastitis cases occurring during the early dry period and at calving (Pankey *et al.*, 1996).

Streptococcus uberis

Streptococci such as *S. uberis* have been reported in the literature as causative agents of mastitis from as early as the late 1920's (Minett *et al.*, 1932). The increased use of management practices to control contagious mastitis pathogens has enabled environmental mastitis pathogens to establish a more prominent profile (Bennett, 1990; Godkin, 1997). Mastitis caused by *S. uberis* appears to be increasing in dairy cattle populations nation-wide (Bramley and Dodd, 1984; Cullen, 1969; King, 1981; Oliver, 1988; Watts, 1988; Oliver *et al.*, 1990). During the 1940's to the late 1960's reports of *S. uberis* mastitis infection prevalences ranged between 3% and 12% of the cases reported (Slanetz and Naghski, 1940, Packer 1947, Muck 1964, Dhanda 1962, and Walsh 1968). In 1980, *S. uberis* accounted for 17-20% of the mastitis cases reported in the United Kingdom (Wilson and Kingwill 1975). A more recent article (Hillerton *et al.*, 1993) reports that 33% of the clinical cases in the UK were caused by *S. uberis*. In New Zealand, *S. uberis* was one of the main causes of mastitis in 1992 (MAF, 1992). It constituted 33% of the cases reported, while *S. aureus* constituted 54% of the cases.

Ecology of *Streptococcus uberis*

The ecology and pathogenesis of *S. uberis* is not as clearly mapped out as the ecology of other major mastitis pathogens. *Streptococcus uberis* is deemed to be an environmental pathogen because it resides in the environment where the cow lives (Smith and Hogan, 1993). It is a ubiquitous organism with a widespread distribution in the cow's environment, as well as in extramammary and intramammary sites (Cullen, 1966; Dodd and Neave, 1970; Newbould, 1975; Bramley *et al.*, 1978 and 1979; Smith *et al.*, 1985). There is conclusive agreement that *S. uberis* is an environmental pathogen (Cullen 1966, Dodd and Neave,1970, Newbould 1975, Bramley *et al.*,1978 and 1979); although it is characterised as both a contagious and an environmental pathogen. There is considerable controversy on the primary reservoir of *S. uberis*. *Streptococcus uberis* is a normal inhabitant of the cow's intestinal tract (Kruze and Bramley, 1983) and has been cultured from a multitude of extramammary sites on cows with and without *S. uberis* mastitis. *Streptococcus uberis* has also been involved in other diseases of cattle including vaginitis, metritis, endocarditis, and abortion (Buddle *et al.*, 1989). *Streptoccus uberis* has been isolated from the ventral abdomen (Razavi-Rohani and Bramley, 1981; Cullen, 1966), the outside flank and shoulder (Bramley *et al.*, 1979), the lips (Bramley *et al.*, 1979; Cullen, 1966; Sharma and Packer, 1970) and from the udder and teat skin (Buddle *et al.*, 1989; Bramley *et al.*, 1979; Sharma and Packer, 1970; Bramley, 1984; Cullen, 1966; Sweeney, 1964) of cows during different seasons and stages of lactation. Not all investigators are in agreement about the common extramammary site of *S. uberis* colonisation in cows.

The order of importance of the isolation sites varied among the herds studied. Cullen (1966) reported the ventral abdomen and the lips as common sites for isolation of *S. uberis,* even when the incidence of positive *S. uberis* faecal samples was low. Sharma and Packer (1970) and Bramley *et al.* (1979) confirmed the later study, and concluded that the lips were a common isolation site of *S. uberis* organisms in cows from a US herd and herds in the UK. Bramley *et al.* (1979) concluded that although *S. uberis* was cultured from many sites, with the belly being one of the less important ones, none of these were primary sites as sources of *S. uberis* infection.

Virulence factors of *Streptococcus uberis:*

Clinical and subclinical cases and even acute systemic cases of *S. uberis* mastitis have been reported. Oliver *et al.* (1997) outlined the multitude of potential virulence factors for *S. uberis*, which enable it to successfully, cause mastitis in the lactating and/or non-lactating mammary gland. These mainly are proteins that may or may not be present in a virulent form of *S. uberis* causing a case of mastitis. These proteins may assist *S. uberis* to adhere to the mammary epithelial lining and/or avoid phagocytosis by inflammatory cells (Leigh and Field 1994).

Phagocytosis

Streptococcus uberis has the greatest resistance to phagocytosis by bovine mammary polymorphonucleocytes (PMN's) when compared to other mastitis pathogens (Leigh and Field, 1994). The antiphagocytic function of the hyaluronidase capsule can act as a physical barrier between the *S. uberis* and the cow's cellular defence mechanisms (Almeida *et al.*, 1992; Almeida and Oliver 1993a and 1993b; Densen and Mandel, 1980; Speert *et al.*, 1988; Spitznagel 1983), but only about 50% of the *S. uberis* isolates are encapsulated (Matthews *et al.* 1994c). Some *S. uberis* can have both cellular and/or extracellular capsular material (Almeida and Oliver, 1993a). The extracellular capsule material also consists of hyaluronic acid of lower molecular weight than the cellular capsule. Although there are virulent non-encapsulated forms of *S. uberis*, the encapsulated forms are more resistant to phagocytosis than the non-encapsulated forms (Almeida and Oliver, 1993b). The Fc receptors on the phagocytic cells can be blocked by hyaluronic acid inhibiting the opsonization activity of the bovine mammary macrophages (McNeil and Wiebkin, 1989). Leigh and Field (1994) noted no significant difference in the binding of IgG_2 to encapsulated and non-encapsulated *S. uberis* microorganisms. Leigh (1994b) contends the 500 - 1000 Dalton molecule contained within the capsular matrix is important to the antiphagocytic nature of the encapsulated *S. uberis*. This latter molecule deemed a neutrophil toxin (Leigh 1994b) could inhibit and/or lyse bovine neutrophils.

Two proteins, which possess antiphagocytic activity, are called the M and R-like proteins. The R-like protein is a 65 kDa protein on the cell surface of some streptococci and has been reported in *S. uberis* isolates (Groschup and Timoney, 1993). The M-like protein is also a cell associated protein in streptococci which can inhibit complement activity of the host cells and mask bacterial epitopes by binding extracellular matrix proteins (Fischetti, 1989). M-like protein can contribute to the adherence of streptococci to mammary epithelial cells (Hollingshead *et al.*, 1993; Wang and Stinson, 1994).

Adherence and invasion

It was speculated that *S. uberis* organisms reside in the lumenal areas of the secretory alveoli and ductular tissue (Anderson, 1988; Thomas *et al.*, 1994). *Streptococcus uberis* adheres to or invades mammary epithelial cells or it will be flushed out during milking. As mentioned above, *S. uberis* can possess components, which may assist the organism to adhere to the epithelial lining of the gland. *Streptococcus uberis* can attach to mammary epithelial cells, although this adherence is not as strong as the adherence of *S. agalactiae* and *S. aureus* (Frost *et al.*, 1977; Ditcham *et al.*, 1996). Thomas *et al.* (1992) concluded that *S. uberis* can not adhere to healthy mammary epithelial cells *in vitro*. The microorganism did adhere to endothelium coated with fibrin. Almeida *et al.* (1996) showed an affinity of *S. uberis* to bind to laminin, collagen, and fibronectin. The encapsulated forms adhered significantly better to the mammary epithelial cells, laminin, collagen, and fibronectin than did the nonencapsulated forms. During

lactation, a gland with fibrosis or a gland with a chronic infection would be an ideal environment for the establishment of a *S. uberis* infection.

Streptococcus uberis can invade mammary epithelial cells as can *S. aureus* and *S. dysgalactiae*, but this process requires protein synthesis (Matthews *et al.*, 1994a). *Streptococcus uberis* has been shown to invade mammary cells as early as 18-24 hours after infection (Matthews *et al.*, 1994a; Thomas *et al.*, 1994).

Uberis factor

The uberis factor was described first by Skalka (1980). Little is known about the importance of the uberis-factor with regards to its potential role in virulence, except that this 42 kDa protein is thought to be chemically similar to the CAMP-factor of *Streptococcus agalactiae* (Lopes *et al.*, 1995).

Housing

Housing facilities, bedding material and loafing areas, if poorly designed and managed will increase the exposure of cows to ES (Faull *et al.*, 1983; Smith and Hogan, 1993; Smith and Hogan, 1995). Bacterial counts are lower in inorganic material, such as sand, compared to organic material (Bramley and Dodd, 1984; Hogan *et al.*, 1989a). Straw bedding is an excellent source of *S. uberis* organisms (Erskine *et al.*, 1987).

Although New Zealand cows are kept on pasture, they are not exempt from being infected by environmental pathogens. Certain conditions can lead to high levels of ES exposure of pastured animals (Harmon *et al.*, 1992). Conditions which cause decreases in the amount of grass cover (Kg of dry matter per hectare) could lead to increased exposure to ES in pastured animals, for example, overgrazed paddocks resulting from high stocking rates, muddy areas after a heavy rain or areas under a shady tree.

The general belief is that New Zealand cows pick up *S. uberis* from paddocks heavily contaminated with faeces. *S. uberis* has been isolated from rectal swab samples taken in herds known to have *Streptococcus uberis* infection, but the isolation numbers are frequently low to nil (Cullen 1966; Bramley *et al.*, 1979). Cullen (1966) concluded that faeces are not an important source of *S. uberis.* Bramley (1982) cultured *S. uberis* from 34% of the faecal samples collected from cows in the UK kept on straw bedding. The average isolate numbers in the faecal samples were low compared to the high levels of *S. uberis* found in the straw bedding (as high as one million cells in a kilogram of straw in some cases). Bramley (1982) concluded that although *S. uberis* may be present in faeces, it is not the primary source of *S. uberis* infections.

Seasonal variation

Hogan *et al.* (1989) reported that there was no seasonal variation in streptococcal mastitis in herds with low somatic cell counts. In contrast to this study, seasonal variation in ES (Hogan *et al.*, 1989a; Hogan *et al.*, 1989b; Smith *et al.*, 1985; Smith *et al.*, 1985b), in particular *S. uberis* mastitis was noted in several other studies (Robinson *et al.*, 1985; Cullen and Little 1969; Bramley *et al.*, 1979; Hughes 1960; Sharma and Packer, 1970; Buddle *et al.*, 1989; Berger and Francis, 1951; Hughes 1960; Sweeney 1964; Todhunter *et al.*, 1994). The majority of the studies reported an increase in the cases of *S. uberis* mastitis during the winter months (Robinson *et al.*, 1985; Bramley *et al.*, 1979; Hughes 1960; Sharma and Packer, 1970; Buddle *et al.*, 1989; Berger and Francis,1951; Hughes 1960; Sweeney 1964; Todhunter *et al.*, 1994), particularly during wet conditions (Cullen and Little 1969). In a study involving cattle housed in the winter, Robinson *et al.* (1985) reported a high incidence of *S. uberis* mastitis during the winter and summer months, with the incidence being highest during the winter. Indoor housing environments can be controlled during the winter, unlike pasture conditions. If "wet" conditions exist indoors, then there is a problem with crowding and/or management of housed cows. The high frequency of *S. uberis* isolation from mastitis cases in summer is in contrast to the results of an earlier study (Stuart *et al.*, 1951), but agrees with a recent study that reported a higher rate of *S. uberis* mastitis during the summer than the winter (Todhunter *et al.*, 1994). Todhunter *et al.* (1994) did not assess why an increased rate of *S. uberis* infections occurred in summer. Robinson *et al.* (1985) assumed that flies may be an important vector for the spread of *S. uberis* mastitis during the summer, because *S. uberis* has been isolated from flies.

Stage of lactation

In very early studies, Minett *et al.* (1932) and Ineson and Cunningham (1949) concluded there was no change in infection rate at different stages of lactation. In later trials, in both non-seasonal and seasonal herds, the stage of lactation did affect the isolation incidence of *S. uberis* (Robinson *et al.*, 1985; Todhunter *et al.*, 1994; Bramley *et al.*, 1979; Oliver *et al.*, 1956; Sharma and Packer, 1970) and other pathogens (Oliver, 1988). King (1981) reported an increase in *S. uberis* mastitis in unmilked cows, which would correspond to the dry cow period (winter months in spring calving herds). This has subsequently been shown to being the most favourable time for *S. uberis* infections (Robinson *et al.*, 1985; Todhunter *et al.*, 1994). In New Zealand, it could be the wet muddy conditions of the winter (Cullen and Little 1969) which affects the rise in infection during the early dry period through to early lactation. Others suggest that the absence of milk being flushed out of the udder (Frost *et al.*, 1977; Smith *et al.*, 1985b), changes in mammary secretion at dry off (Marshall *et al.*, 1986; Todhunter *et al.*, 1985), and/or a lack of a keratin plug at dry off (Comalli *et al.*, 1984; Eberhart, 1986; Williamson *et al.*, 1995) may affect the incidence of infections during the early dry period. Cows lacking a tight seal of the keratin plug will leak milk between milkings, at dry off, and in the periparturient period. Open streak canals will be susceptible to invasion by pathogenic organisms. Schukken *et al.* (1993) noted cows to be at a 4 times greater risk of clinical mastitis during the dry period if they leaked milk. The lactoperoxidase-thiocyanate-hydrogen peroxide system (LDS) is a mammary gland antibacterial mechanisms used to rid the mammary gland of bacterial

infections, but this mechanism has limited activity against *S. uberis* in the nonlactating gland. (Marshall *et al.*, 1986).

At dry off, the incidence of mastitis may be affected by the amount of milk produced just prior to dry off, infection status at dry off, the use of DCT and the type of drying off technique used (Natzke *et al.*, 1974; Oliver *et al.*, 1956d). Oliver *et al.* (1956d) noted no difference in the rate of new dry period infections when cows were dried off by either the intermittent milking or the stop method. In a similar study cows uninfected at drying off, dried off by the "stop" method, had a significantly higher incidence of mastitis at dry off compared to cows dried off using an intermittent milking procedure (Oliver *et al.*, 1956c). Newbould and Neave (1965) noted a higher rate of infection in cows milked once a day as opposed to twice daily prior to dry off. A greater number of infections were noted in the cows which had the highest milk yield at dry off (Oliver *et al.*, 1956d; Neave *et al.* 1968). Neave *et al.* (1968) specifically noted that cows producing ≥ 9 kg/day at the time of drying off developed more new infections than cows which produced less milk on the same drying off date.

Hillerton *et al.* (1995) noted 87% of all new IMI occurred between dry off and 7 days into the following lactation. Early lactation is a common period for an increased incidence of mastitis caused by *S. uberis* (Sharma and Packer, 1970) and other organisms as well (Ward and Castle, 1944; Withers, 1955; Murphy and Stuart, 1944; Oliver *et al.*, 1956b; Hogan and Smith, 1997; Smith *et al.*, 1985b). Erb *et al.* (1984) reported that the incidence of clinical mastitis was 4 times higher during the first 15 days of lactation than at any other period during that lactation. Oliver (1956b) reported

a negative correlation between rate of first infection and days in milk during a given lactation.

Age

The age of cows is another factor, which plays an important role in the incidence of *Streptoccus uberis* infections. Many investigators report a positive correlation between the incidence of udder infection and age (Bendixen, 1935; Klein and Learmonth, 1935; Gill and Holmes, 1978; Hopkirk *et al.*, 1943; Ward and Castle, 1944; Murphy, 1945; Arthur, 1947; McEwen and Cooper, 1947; Schalm and Ormsbee, 1949; Hughes, 1954; Withers, 1955; Macmillan *et al.*, 1983; Smith *et al.*, 1985; Todhunter *et al.*, 1995). Sharma and Packer (1970) reported a specific increase in the prevalence of *S. uberis* infection with increasing age of the cow. The increase in infection in the older cows may be a consequence of a higher pre-existing infections rate in the older cows resulting in an increasing reinfection rate (Oliver *et al.*, 1956b). Robinson *et al.* (1985) confirms the latter study by concluding that clinical cases of *S. uberis* mastitis during the lactation period were mainly from those quarters which had been infected subclinically with *S. uberis* at calving. Older cows produce more milk than younger cows, which may contribute to a higher incidence of mastitis in older cows, because higher producing cows are more susceptible to mastitis (Shook, 1993).

Diet

A poor diet can affect the ability of the cow's natural immune system to defend against mastitis pathogens (Smith *et al.*, 1984b; Smith and Hogan, 1993; Smith and Hogan, 1995). Cows, which are deficient in vitamin E and/or selenium are at a greater risk of environmental mastitis (Shook, 1993).

Mastitis prevention measures

Preventative medicine is the preferred means of dealing with mastitis. Mastitis prevention measures are directed at minimising bacterial exposure of the gland and enhancing the immunity of cows. In general, decreasing exposure levels require improved hygiene in the cows' environment and the culling of older cows with chronic infections. Separating heifers from the other cows in the herd during calving, calving in paddocks with little mud and heavy grass cover (Kg DM/Ha), or a combination of the two with or without DCT in the previous season are common management practices adopted by New Zealand dairy farmers to lessen the exposure of the animals to mastitis pathogens. More grass and less manure covered paddocks undoubtedly would decrease the exposure of the gland to any faecal contaminants.

DCT and teat disinfecting

A survey extending from 1984 through 1994 on 67 farms in the USA revealed that ES were the most frequently isolated organism at dry off (Hogan and Smith 1997). Post-milking teat disinfection (Eberhart *et al.*, 1983; Bramley and Dodd, 1984; Smith *et al.*, 1985; Erskine *et al.*, 1987; Hogan *et al.*, 1987; Pankey and Dresher, 1993; Smith and Hogan, 1993; Bramley 1997) and DCT were reported to have a minimal effect on the incidence of ES infections (Natzke 1981; Kruze and Bramley 1982; Dodd 1983; Eberhart *et al.* 1983; Smith *et al.*, 1985), in particular *S. uberis* mastitis (Pankey *et al.*, 1982; Oliver and Sordillo, 1988a; Oliver *et al.*, 1989b; Hillerton *et al.*, 1995; Williamson *et al.*, 1995). In contrast, other authors report cows which were teat dipped post milking had less ES mastitis than those not dipped (Eberhart *et al.*, 1983; Bramley and Dodd, 1984; Erskine *et al.*, 1987; Oliver *et al.*, 1991). The long duration barrier dips have been effective at controlling ES mastitis (Oliver *et al.*, 1989b; Smith and Hogan, 1993; Hogan *et al.*, 1995). Herds which were teat dipped postmilking and treated with DCT at drying off had a 72% lower rate of infections caused by *S. uberis* in the early dry period than cows which were selectively dry cow treated and not teat dipped post-milking (Robinson *et al.*, 1985). Other authors (Eberhart, 1986; Hogan *et al.*, 1994b; Smith *et al.*, 1985b; Williamson *et al.*, 1995) agree that DCT is generally effective in decreasing the incidence of ES mastitis in the early dry period. Blanket DCT will cure 84% to 100% of ES infections (Bramley 1984; Hillerton *et al.*, 1995) and will reduce the prevalence of ES at the start of the following lactation (Smith *et al.*, 1985; Williamson *et al.*, 1995). Smith *et al.* (1985) specifically noted a reduced rate of *S. uberis* IMI at drying off, but no effect on the incidence *S. uberis* IMI during

the periparturient period. Macmillan *et al.* (1983) determined a higher incidence of clinical mastitis in herds that were blanket treated rather than selectively treated.

Milking hygiene

Poor milking hygiene and malfunctioning milking machines can contribute to ES infections (Pankey *et al.*, 1987; Galton *et al.*, 1988; Pankey, 1989; Pankey and Drechsler, 1993). Hygienic milking habits such as milking only clean dry udders, avoiding contamination of cows from the milkers' hands and milking equipment and milking known infected cows last are preventive measures that can be readily adopted by New Zealand dairy farmers. Pre-milking teat disinfection, a practice not commonly adopted by the average New Zealand dairy farmer, has been reported to reduce the rate of *S. uberis* mastitis (Oliver and Smith, 1982).

Prepartum therapy

Prepartum antibiotic treatment may be of economic benefit in herds with a problem of early lactation mastitis, especially in heifers. Nickerson *et al.* (1995) decreased the level of ES infection by 93% in prepartum heifers, by treating all of them with antibiotics <60 days prior to parturition. This management strategy could be incorporated into a mastitis prevention programme in a New Zealand dairy herd, because heifers usually calve within a 6-week period. The heifers could be treated with antibiotics at one time to minimise labour cost. Pankey *et al.* (1982) decreased *S. uberis* infections by 94% in cows treated 1-3 days prepartum with intramammary antibiotics.

Current intramammary antibiotics are not labelled for the treatment of non-lactating glands except for dry cow therapy products. The use of prepartum antibiotics would require the testing of the milk postpartum for drug residues to minimise the risk of contaminating the milk. The cost of the antibiotics used, labour, testing for drug residues and the risk of increasing infection in those animals treated with intramammary antibiotics should not outweigh the benefit of prepartum antibiotic treatment if such a regime is to be used.

Vaccination

Enhancing the immune response to mastitis can be achieved through genetic selection and vaccination. It is clear that the environment serves as an important source of *S. uberis*; therefore the environment could be targeted in mastitis prevention control programmes. Hygienic milking, pre-milking teat disinfecting and/or vaccinating are preventative available to prevent *S. uberis* mastitis.

There have been some successes and failures with mastitis vaccines over the decades. Bacterial components used for developing vaccines include killed bacterial cells, isolated bacterial cell walls, killed cell-toxoids, toxoids, inactivated bacteria, attenuated bacteria, and live bacteria (Colditz and Watson, 1985). Efficacious vaccines directed towards mastitis pathogens are able to decrease the neutrophil count in vaccinated cows (Howell *et al.*,1956), decrease the rate of new infections in the dry period, and alleviate the severity of clinical signs caused by the particular organism during lactation (Nickerson *et al.*,1993; Nordhaug *et al.*, 1994; Sears *et al.*, 1990; Watson and Schwartzkoff, 1990; Yoshida *et al.*, 1984). *Escherichia coli* vaccines have similar effect, by decreasing the proportion of cows showing clinical disease (Hogan *et al.*, 1992a) and the severity of clinical signs (Hogan *et al.*, 1992b).

Bacterin-toxoid vaccines against streptococci and staphylococci have been shown to be ineffective at decreasing the rate of new infections rate or the severity of clinical signs (Mellenberger, 1977). Rabbits immunised with staphylococcal alpha toxin were

protected against the acute lethal haemorrhagic form of mastitis, but not the chronic form (Anderson, 1978). The efficacy of a vaccine will depend upon several factors including, the type of vaccine used, the timing of vaccination (stage of lactation) and the natural immunity of the individual animal (Mellenberger, 1977).

Howell *et al.* (1956) subcutaneously vaccinated cows six times over a nine week period against *S. agalactiae* mastitis and noted that vaccinated cattle cleared infections caused by *S. agalactiae* more frequently and had an overall lower neutrophil count than the unvaccinated cattle. No significant difference was noted between the vaccinated and non-vaccinated cattle with regards to resistance to the initial bacterial challenge. The investigators did note a persistent immunity in those animals that were rechallenged several months later. Vaccinating against S. agalactiae mastitis decreased the rate of new infections and lessened the clinical state (Bracewell and Pattison, 1958; Mellenberger, 1977). Bracewell and Pattison (1958) used only heifers in their trial, which would minimise the confounding effect of the naturally higher titres against *S. agalactiae* mastitis found in older animals.

In a more recent trial (Leigh, 1994b), a group of cows were initially vaccinated against *S. uberis* and then challenged with the same strain. They exhibited higher milk yields, lower SCC and shed fewer organisms than the unvaccinated cows. The latter trial like several other trials (Erskine *et al.*, 1988; Hill, 1988a; Finch *et al.*, 1997) have exhibited a positive response to vaccination against streptococci, but these effects were seen in vaccinated animals challenged by the same strain against which they were vaccinated. Hill (1988a) artificially induced *S. uberis* mastitis in individual quarters, and

subsequently challenged the cows 27 - 105 days later. He challenged different quarters on some of the same cows that had infections from the initial challenge. Eighty seven percent of the quarters developed clinical mastitis from the first challenge and only 32% developed clinical mastitis from the second challenge. A significant decrease in infection incidence ($X^2 = 19.3$; $p < 0.001$). This indicated that an infection in one quarter of a cow by a particular *S. uberis* strain can produce a protective effect in other quarters of that same cow, if that cow is subsequently challenged by the same strain.

Different strains of bacteria respond in different ways to an individual vaccine (Slanetz *et al.*, 1963; McDowell, 1974). The numerous strains of *S. uberis* and its encapsulated form, which diminishes the phagocytic activity of the neutrophils, has hindered the development of a efficacious vaccine to be used against its various strains.

The herd response will depend on the prevalence of the specific organisms causing mastitis infection. Watson *et al.* (1996) vaccinated 7 Australian herds against *S. aureus* mastitis. Cows were given their first bacterin toxoid staphylococcal vaccination at dry off and heifers were vaccinated during their last trimester of pregnancy. A booster was administered 4-6 weeks later. The prevalence of *S. aureus* infections was lower in the vaccinated animals than in the controls, naturally exposed to pathogenic bacteria, but the difference was not statistically significant for the pooled 7 herds. The vaccinated animals in one of the herds with a high prevalence of *S. aureus* infections had significantly fewer clinical and subclinical infections than the controls. When only the heifers in the latter herd were considered, the prevalence of subclinical mastitis between the vaccinated and control group was significantly lower in the vaccinates, but the difference was not noted with clinical mastitis in these

groups. The authors concluded that the vaccine might be efficacious in herds with a high prevalence of *S. aureus* mastitis but not in herds with a low prevalence.

The timing of vaccination may effect the outcome (Mellenger, 1977). Giraudo *et al.* (1997) vaccinated one group of heifers 8 and 4 weeks prepartum and another group received two vaccinations, at one week and at five weeks postpartum. The vaccine contained *S. aureus* exopolysaccharides and inactivated, unencapsulated *S. aureus* and *Streptococcus* spp. cells. The 10 control cows received a placebo 8 and 4 weeks prepartum. The frequency of intramammary infections by *S. aureus* was reduced by approximately 12% in both of the vaccinated groups compared to the control group. The frequency of latent *S. aureus* infections was lower in vaccinates. They concluded that vaccination prepartum or postpartum had similar effects on the frequency of *S. aureus* infections, which is contrary to what other researchers have noted (Oliver *et al.*, 1956b; Munch-Peterson 1968). Calozolari *et al.* (1997) also vaccinated cows greater than 7 months pregnant on the initial vaccination and 4 weeks later, with *S. aureus* exopolysaccharides, inactivated and unencapsulated *S. aureus* and *Streptococcus* spp. cells. They reduced subclinical and clinical mastitis in the vaccinated animals by 44% and 66% respectively compared to the controls and there were fewer latent infections in the vaccinated animals (Calozolari *et al.*, 1997).

The greatest milk production loss (monetary loss) occurs with subclinical infections (Fetrow, 1983). There was no difference in loss of milk production between the vaccinated and non-vaccinated animals, but they did note a positive correlation between the increase in the challenge dose and increasing milk loss (Howell *et al.*, 1956). Hill (1988a) noted a diminished effect in milk loss during the second challenge

compared to the first. Milk yield dropped by nearly 50% within approximately 24 hours after the first challenge, but only 24% after the second challenge. The most effective and economical vaccine will be one which will mount a response against subclinical mastitis (Anderson, 1978). During a subclinical mastitis episode there is minimal tissue damage and less movement of blood constituents into the mammary gland. Vaccine failure is due in large part to the failure of the antibodies to penetrate the blood - milk barrier (Colditz and Watson, 1985).

The most recent topic of discussion with regards to possible development of a vaccine is the ability of *S. uberis* to convert bovine, ovine or equine plasminogen to plasmin (Leigh 1993 and 1994). The plasmin degrades the milk casein providing an amino acid source for the multiplication of the *S. uberis* organisms (Leigh, 1993). The plasminogen activator (PauA) is approximately 30 kDa in molecular weight and has been isolated from at least 90% of the clinical *S. uberis* cases in the UK (Leigh, 1997). Prevention of the plasminogen conversion may retard the growth of *S. uberis*, allowing time for the natural immune system to mount a response and eliminate the infection. Quarters from animals immunised with the total (100 μg/ml) PauA antigen shed less bacteria after an experimental challenge of *S. uberis* than those immunised with a depleted (0.1 μg/ml) amount of PauA antigen (Leigh, 1997). These animals were vaccinated subcutaneously 5 times during a 10 week period and were not challenged until 16 weeks into lactation (3 weeks after the last vaccination). Cows in their 4^{th} month of lactation are at a lower risk of *S. uberis* mastitis regardless of vaccination. In New Zealand a vaccine would be advantageous, if injections could be given that provide protection in early lactation and during the first few weeks of the dry period. This is when the incidence of *S. uberis* mastitis is high (Sharma and Packer, 1970;

Robinson *et al.*, 1985; Todhunter *et al.*, 1994; Pankey *et al.*, 1996). There is no available streptococcal vaccine on the market to use as an effective preventative measure against *S. uberis* mastitis in New Zealand.

Enhancing involution and intramammary devices

Researchers have tried to accelerate the involution process by inserting intramammary devices into the teat sinus via the streak canal to decrease dry period infections caused by *S. uberis*. No significant difference in the incidence of mastitis was shown between cows infused with concanavalin A and phytohemagglutinin into the mammary gland and those not treated, when glands were challenged 7 days post-treatment (Maki and Oliver, 1987) with *S. uberis* organisms. Intramammary devices did provide some protection against *S. uberis* infections, by preventing their establishment in the gland (Buddle *et al.*, 1989).

SAMM Plan

The SAMM Plan is a 5 stage prevention programme first developed in 1990 by the Dairying Research Corporation in conjunction with the Livestock Improvement Corporation of the New Zealand Dairy Board, as a means to improve milk quality nation-wide, by providing farmers with recommended management strategies to reduce the incidence of mastitis in their herds. The SAMM Plan was born from the 5-

point plan, which had been developed in the United Kingdom, a country where non-seasonal calving predominates. The main 5 points of the 5-point plan, namely check milking equipment regularly, prompt treatment of all clinical cases, efficient and effective teat spraying, dry cow therapy (DCT) and culling chronically infected cows are included in the SAMM Plan but with respect to seasonally calving herds managed on pasture. The suggestions in the Plan were initially based on research from other countries. As research is done in New Zealand, this will be incorporated in the plan together with the information obtained from other countries. A large proportion of the recent SAMM Plan (1996-97) is from information derived in New Zealand.

Identification of subclinical mastitis:

Electrical conductivity

Electrical conductivity meters detect changes in milk conductivity associated with changing infection status. The concentrations of sodium and chloride ions in milk from infected quarters are increased, thereby increasing the conductance of the milk (Davies, 1938). Several different hand held devices and systems incorporated into the milking machine (in-line) are available to the New Zealand dairy farmer. The in-line detection systems which measure the average EC of a particular cow's milk during several consecutive milkings is the most accurate means to measure conductivity, achieving sensitivities in detecting mastitis of 33 - 77% (Nielen *et al.*, 1997). The

economics of having an in-line EC meter on an average New Zealand commercial herd has presently not been reported.

The conductance of the milk can be affected by the cow's age, her SCC (Davis, 1975; Greatrix *et al.*, 1968), milk production level (Chamings *et al.*, 1984) stage of lactation, stage of the milking process (Gebre-Egziabher *et al.*, 1979; Hillerton and Walton, 1991), and infection status (Gebre-Egziabher *et al.*, 1979; Hillerton and Walton, 1991). Chamings *et al.* (1984) noted a significant difference between in the EC readings between herds. Although the EC and SCC do not always coincide (Jones, 1949; Little *et al.*, 1968), the EC value will be affected by the SCC and the presence of bacteria (Chamings *et al.*, 1984; Hillerton and Walton, 1991). Chamings *et al.* (1984) reported a normal secretion (SCC < 500,000 cells/ml and no bacteria cultured) to have a value of approximately 5.79 mS/cm or less, a latent infection (SCC < 500,000 cells/ml and bacteria cultured) of 5.79 mS/cm to 6.35 mS/cm, non-specific infections (SCC > 500,000 cells/ml and no bacteria cultured) of between 6.35 mS/cm and 7.02 mS/cm and a definite mastitis case (SCC > 500,000 cells/ml and bacteria cultured) to be greater than 7.02 mS/cm. The EC values considered normal on some herds were abnormal on other herds. Others reported normal quarters to be 5.4-5.6 mS/cm (Hillerton and Walton, 1991), <5.79 mS/cm (Chamings *et al.*, 1984), and <7.5 mS/cm (Gebre-Egziabher *et al.*, 1979). Infected quarters were noted to have EC readings of >7.02 mS/cm (Chamings *et al.*, 1984) and >8.0 mS/cm (Gebre-Egziabher *et al.*, 1979).

Researchers have advocated the use of EC as a screening test for subclinical mastitis (Fernando, *et al.*, 1985; Hillerton and Walton, 1991; Little *et al.*, 1968). Fernando *et al.* (1985) found that the EC was more accurate than other indirect tests (sodium,

chloride, potassium, lactose, Bovine serum albumin-BSA, and SCC) at detecting subclinical mastitis. Greatrix *et al.* (1968) reported that EC is most effective at detecting abnormal milk when the SCC is between 500,000 - 5,000,000. Chamings *et al.* (1984) agrees that the EC is a good predictor of infection status, but not as good as the California Mastitis Test (CMT) or a direct measurement of somatic cell counts like the results of the Fossomatic (A/S Foss Electric, Hillerod, Denmark).

Electrical conductivity readings and subsequent diagnosis can be affected by the milk temperature, the fraction of the milk sampled (i.e. beginning, middle, end of the milking period), the bacterial infection present, or fat concentration (Davis, 1975; Gebre-Egziabher *et al.*, 1979; Chamings *et al.*, 1984). Increased fat content and cellular content will decrease the EC reading (Davis, 1975; Fernando, 1982; Fernando *et al.*, 1985). The last fraction of milk during a single milking period is expected to have a lower EC reading than the first and middle fractions, because of a higher fat content. This is contrary to what was reported by Gebre-Egziabher *et al.* (1979), who noted an increase in conductance towards the end of the milking process. Fernando *et al.* (1982 and 1985) suggested postmilking strippings are the most accurate. The effects of all these factors must be minimised in order to maximise the EC's ability to detect subclinical mastitis.

Researchers have noted differences in EC caused by the type of bacteria cultured (Chamings *et al.*, 1984), with *S. agalactiae* causing a higher conductance than *S. aureus*. Hillerton and Walton (1991) reported readings between 7.1 - 7.5 mS/cm for quarters infected with *S. aureus*, while *S. uberis* quarters had similar EC readings to the normal quarter readings at 5.3 - 5.6 mS/cm. They concluded that EC was useful at

detecting *S. uberis* infections 48 hours before they culture positive. Electrical conductivity is useful at detecting *S. uberis* infections, but not chronic infections because milk composition changes are negligible in these cases (Milner *et al.*, 1996).

The sensitivity and the specificity of a test will vary among cows and herds. An absolute value for resistance or conductance should not be used when attempting to diagnose whether a cow has mastitis. If quarter samples are taken, then the individual quarter results should be compared to the other quarter readings from the same cow (Davies, 1938; Davis, 1947; Gebre-Egziabher *et al.*, 1979; Greatrix *et al.*, 1968). Gebre-Egziabher *et al.* (1979) determined that a conductance ratio of 1.2 (difference between the quarter with the lowest conductance and another quarter being examined in the same cow) gave the smallest proportion of false negatives and false positives. Fernando *et al.* (1985) reported 16.5% and 42.7%, false positive and false negatives respectively, using EC on foremilk samples. Chamings *et al.* (1984) reported twice as many false positives (33%) from foremilk samples, than the latter study and a high sensitivity of 91%. This falls into the range reported by Greatrix *et al.* (1968) who reported the sensitivity of a hand-held EC meter to range between 53 - 97%. A sensitivity of 57.3% was obtained from an in-line EC system (Gebre-Egziabher *et al.*, 1979). Fernando *et al.* (1985) reported a specificity of 90 - 100% for an in-line system. The latter results suggest that a hand-held or in-line EC system's ability to detect bacterially negative samples is better than its' ability to detect bacterially positive samples.

Somatic Cell Counts:

Somatic cells are mainly inflammatory cells with a small percentage of mammary epithelial cells. In a healthy gland the predominant inflammatory cell is the mammary macrophage, while the neutrophil is the predominant somatic cell in the infected gland. Mobilisation of WBC to the mammary gland is an important immune response to fight infections in the mammary gland (Sischo *et al.*, 1997). An increase in a cow's individual somatic cell counts (ICSCC) is an indication of infection in her mammary gland. Monitoring changes in ICSCC, with subsequent bacterial culturing, is the common way to screen animals for new IMI (Jones *et al.*, 1984).

Measuring somatic cell counts

Somatic cell counts can be measured directly and indirectly. The main direct methods are the Coulter Counter (Ali and Shook, 1980), which counts particles as they flow through an electric field and the Fossomatic[1] (Arave and Albright, 1976), which counts the number of cells that are fluorescing after treatment with a dye. These direct methods are used usually by milk factories and other diagnostic laboratories, which need to process large numbers of milk samples. Indirect means to measure SCC are the CMT, Wisconsin Mastitis Test (WMT) and the Rapid Mastitis Test (RMT); methods that are used in the laboratory and on the farm.

1 A/S Foss Electric, Hillerod, Denmark.

Rapid or California Mastitis Tests

The RMT and the CMT are synonymous tests[2]. An equal amount of the test reagent and the sample are gently mixed in a swirling motion in the wells of specialised paddles. The reagent reacts with the nuclear material of the cells in the milk samples forming a gel-like material of varying viscosity, dependent upon the concentration of cells in the milk sample. The higher the number of cells in the sample the greater the viscosity of the gel formed. The reaction for the RMT or CMT (R/CMT) is scored from 0 (no reaction) to 3 (Figure I). The R/CMT are very subjective tests relying on the experience of the user to detect the presence of gel in the test mixture (milk sample plus the reagent).

2 They are basically very similar test, but marketed under different names. The RMT is marketed by VETPACK in New Zealand. Most information obtained from the literature will be based on using the CMT.

Figure I: Scoring and interpretation of the R/CMT

Scoring	Precipitation	SCC interpretation (x 1000 cells/ml)	Interpretation
Negative(N)	none	0 - 200	normal and healthy
Trace (T)	slight	150 - 400	any thickening, suspicious, test again
1	small amount of precipitate	300 - 1,000	any thickening, suspicious, test again
2	distinct precipitation	700 - 2,000	infected teat
3	gel	> 2,000	heavily infected teat

Modified chart from Dohoo and Meek (1982)

The WMT was developed after the CMT. It too is a rapid and inexpensive test, which works on the same principles as the R/CMT, except the test is done in test tubes as opposed to a paddle. The disadvantages of this test is the lack of ease of use cow-side and the sensitivity of the test to temperature changes of the milk (Thompson, 1970). The milk sample must be kept at temperatures between 0 - 4.4 °C, until ready for testing. Standardised reusable test tubes and caps, pipettes, cannulas and the reagent (a detergent) are needed. A waterbath is required to maintain the reagent at 35 °C, during the test. An equal amount of the reagent is mixed with 2 ml of the sample. The nuclear material of the cells and the reagent combine, forming a viscous solution. The more somatic cells, the greater the depth of the viscosity. The WMT like the R/CMT

can be performed on ICSCC as well as BMSCC. The results are recorded in millimetres (mm) and can be related to the milk production loss (Figure III).

Figure II: Relationship between mastitis tests scores, bulk milk somatic cell counts (BMSCC), and herd milk production losses

CMT	WMT (mm)	BMSCC (x 10^3)	% Production Loss
0	3 - 6	140 - 225	3
T	7 - 10	260 - 380	5
1	11 - 24	420 - 1200	8 - 12
2	25 - 35	1280 - 2280	16 - 20

Modified chart from Philpot (1978)

Quarter samples

The SCC of a healthy animal will vary from cow to cow and from herd to herd. The SCC of an uninfected quarter, with no previous history of infection, is approximately 260,000 (Dohoo and Meek, 1982). Milk yields are noted to begin to decline when SCC from quarter samples exceed 100-150,000 cells/ml (Reichmuth, 1975). The average SCC of quarters with a previous history of infection would be 600,000 cells/ml (Dohoo and Meek, 1982). Klastrup (1975) recommends a much lower cut-off of 300,000 cells/ml for an infected quarter.

Composite samples

A composite sample combines a proportion of milk from each quarter into a single sample for testing. Interpretation of the SCC of a composite sample with regards to infection status is variable. Authors have used thresholds between 250,000 - 500,000 cells/ml (Rindsig 1979; Andrews *et al.*, 1983), with varying levels of false positives and negatives depending on the thresholds used and the prevalence of infection in the herd being tested. A composite sample from a cow with all 4 quarters uninfected will average 113,000 - 251,000 cells/ml depending on the age of the cow (Eberhart *et al.*, 1979). Composite samples from cows harbouring commensal organisms (minor pathogens, i.e. coagulase negative staphylococci and *Corynebacterium bovis*) are between 190,000 - 519,000 cells/ml (Eberhart *et al.*, 1979). Dohoo *et al.* (1981) states that a composite sample with a SCC >228,000 cells/ml should be deemed infected.

Using the latter threshold, approximately 86% of cows will be correctly diagnosed (Dohoo *et al.*, 1981). Variation in the level of SCC has been noted with the type of major pathogen cultured (Ward and Schultz, 1972; Schultz, 1977). Cows with major pathogens (i.e. *S. aureus*, *S. agalactiae*, and other streptococci) on average have counts greater than 600,000 cells/ml (Natzke *et al.* 1972; Ward and Schultz, 1972; Schultz, 1977; Eberhart *et al.*, 1979). The SCC in a composite sample will vary with the number of infected quarters. A doubling affect was noted with each additional quarter infected in a cow (Dohoo and Meek, 1982). The level of SCC that can be expected from a particular infection should be used as a guideline and should not be used as the ultimate means of diagnosis.

Milk yield and SCC:

Jones *et al.* (1984) reported that there is a linear relationship between decreasing milk yield and increasing SCC. In that same study, they reported an increasing infection rate in cows with SCC between 200 to 400 x 10^3 cells/ml. Various quarter production losses have been reported (Figure II). The overall percentage of milk production loss may not change, depending on the age and the number of quarters infected. Woolford *et al.* (1984) reported a compensatory gain in milk production in the uninfected quarters of cows but not in heifers. The 8% production that is lost in the infected quarter of cows is produced in the other non-infected quarters.

Figure III: Percentage of milk production losses per quarter based on R/CMT results.

R/CMT	% Production Loss*
T	2.8 - 9.0
1	11.4 - 19.5
2	25.6 - 33.8
3	43.4 - 50.0

(Philpot, 1967; Forster and Ashworth, 1967; Dobbins, 1977)

Age and SCC:

Somatic cell counts vary with age (Ward and Horton, 1965). Higher SCC are noted in older cows (Beckley and Johnson, 1966; Blackburn, 1966; Daniel *et al.*, 1966; Gill and Holmes, 1978; Schultz, 1977). Older cows will have a greater cellular response to mastitis pathogens (Eberhart *et al.*, 1979; Marshall and Edmondson, 1962).

Stress:

Stressful situations such as pregnancy diagnosis, calving, decreased feed availability and the initial mixing of unfamiliar animals will elevate the SCC level (Kay *et al.*,

1977). In seasonal herds, groups of animals are in oestrus at a given time and cows will tend to mount one another just prior to and during oestrus; however, Guidry *et al.* (1975) did not note an increase in SCC with oestrus activity.

Stage of lactation:

Somatic cells have been reported to rise at calving in uninfected animals and stay at elevated levels until 2 weeks into lactation (Cullen, 1968; Natzke *et al.*, 1972; Reichmuth, 1975). The SCC will decline after the first fortnight, but as the lactation progresses, the SCC will increase near dry off (Beckley and Johnson, 1966; Blackburn, 1966; Schultz, 1977). Other studies report uninfected cows did not have a rise in SCC as the lactation progressed (Eberhart *et al.*, 1979; Natzke *et al.*, 1972). Duitschaever and Ashton (1972) reported a rise in SCC at drying off and Bodolh *et al.*, (1976) reported this rise only after daily milk production falls below 4kg. This rise in SCC with declining milk production in uninfected cows was confirmed in a New Zealand study (Lacy-Hulbert *et al.*, 1995). During an individual milking period differences in SCC are noted, with the highest SCC in the strippings (after milking) and the lowest at the beginning of milking (Cullen, 1967a; Smith and Schultz, 1967; White and Rattray, 1965). When ICSCC testing is done a representative sample is taken during the entire milking period to avoid the bias of differences in the somatic cells concentration from the beginning to the end of the milking period.

Seasonal differences:

SCC is reported to be lowest in the winter and highest in the spring/summer (Macleod *et al.*, 1954; Marshall and Edmonson, 1962; Nelson *et al.*, 1967; Nelson *et al.*, 1969; Bodolh *et al.*, 1976; Wegner *et al.*, 1976).

Teat disinfectant and DCT:

Herds that use mastitis prevention measures have lower SCC. Teat disinfectant is associated with lower SCC (Bodolh *et al.*, 1976; Goodhope and Meek, 1980; Hayward and Webster, 1977; Mein *et al.*, 1977; Moxley *et al.*, 1978; Postle *et al.*, 1971; Schultz, 1977). Herds that use blanket dry cow antibiotic therapy have lower SCC compared to herds that use selective treatment (Mein *et al.*, 1977); although there are some contradictory reports (Bodolh *et al.*, 1976; Schultz, 1977).

Bulk milk somatic cell counts:

Bulk milk SCC (BMSCC) is the somatic cell count from a sample from the vat and presents a broad estimate of the overall udder health status for the herd. The BMSCC can be elevated by only a few cows with high ICSCC. The BMSCC can be used as an indicator of infections and milk quality. High BMSCC milk has a lower content of fat,

solids-not-fat, lactose, and casein and higher sodium, chloride, free fatty acids, albumin, and immunoglobulins (Everson, 1980; King, 1972; King, 1978; Roussel *et al.*, 1969; Schultz, 1977).

Bulk milk SCC are measured regularly (daily to fortnightly) for New Zealand dairy farmers. The more frequent readings will be of greater use for monitoring health status. The correlation between BMSCC and the percentage of infected quarters was reported to be between 0.5 - 0.6 (Postle *et al.*, 1971; Reichmuth, 1975; Westgarth, 1975). The BMSCC can not determine the prevalence of infection in a herd, but it can provide a good indication of udder health in a herd. The Livestock Improvement Corporation (LIC) in New Zealand provides the following data for the estimation of infected cows in the herd based on the BMSCC:

Figure IV: The estimated (est.) infected animals in the herd relative to the BMSCC

BMSCC (cells/ml)	est. of % of cows with mastitis
100,000	20
500,000	46
900,000	54

Bacteriological/Serological/Microbiological (Molecular) diagnosis of *S. uberis*:

It is important to first determine if an animal is infected, then the type of infective organism, in order to initiate the correct treatment and/or preventive measures. The EC and SCC are shown to be effective, quick tests for determining the presence or absence of subclinical mastitis, but for final diagnosis these tests should be supported by bacteriological culturing (Gebre-Egziabher *et al.*, 1979; Cullen 1965).

Streptococcus

The three major Gram-positive, catalase negative streptococci causing mastitis in New Zealand are *S. agalactiae*, *S. dysgalactiae* and *S. uberis*. Streptococci are grouped into 4 distinct groups: pyogenic, viridans (called "oral" streptococci), lactic streptococci, and enterococci (called faecal streptococci) (Bridge and Sneath, 1982). Bridge and Sneath (1982) grouped *S. uberis* with *S. agalactiae* and *S. dysgalactiae* in the pyogenic group. From a previous study it was concluded from DNA/DNA hybridisation that *S. uberis* was not closely related to these other common streptococcal organisms causing mastitis (Garvie and Bramley, 1979).

Identification:

Identification of S. uberis

A quick, inexpensive and accurate test is required for clinical identification of the infective organism, so that the correct therapy can be instituted as early as possible.

Streptococcus uberis was first described by Dierhofer in the early 1930's, who it as a minor pathogen, which caused mild or transient mastitis, with only a slight change in the milk composition (Dierhofer, 1930a and 1930b). Up until the 1960's *S. uberis* was combined with the non-*S. agalactiae* or the environmental streptococci group. On closer examination of the *Streptococcus* spp. isolates causing mastitis, *S. uberis* was noted to be the prominent species isolated (Cullen, 1967b). In further studies of *S. uberis* two distinct types (I and II) based on DNA hybridisation were noted (Garvie and Bramley, 1979). Collins *et al.* (1984) confirmed that type I and II had different DNA-DNA hybridisation. Although genetically distinct, phenotypically the two groups were indistinct (Garvie and Bramley, 1979). Williams and Collins (1990) designated *S. uberis* II as *S. parauberis* based on the difference in the nucleotide sequencing of the 16S ribosomal RNA.

Biochemistry of S. uberis

Streptococcus uberis is a heterogeneous species (Mellenberger, 1977; Hardie, 1986). Its' consistent characteristics are that it is a Gram-positive, catalase-negative, coccus which ferments carbohydrates to lactic acid as the primary end product. *S. uberis* is grouped in the Lancefield E group (only about 20% of the isolates) and can be alpha-haemolytic or non-haemolytic with variable biochemical and serological characteristics (Cullen, 1967b). Cullen (1967b) stated the fermentation of trehalose, mannitol, lactose, and salicin are most reliable tests for identification of *S. uberis*, although Miller and Heishman (1940) reported *S. uberis* isolates which did not ferment trehalose. Watts (1988b) noted that *S. uberis* I did not utilise sucrose, whereas 100% of the *S. uberis* II isolates did.

Ideally for identification purposes the selected biochemical test will give consistent results for the organism being examined. *S. uberis* are typically hippurate, aesculin, and pyroglutamic positive, but are bile-aesculin negative and unable to grow in 6.5% NaCl broth. Different culturing and laboratory techniques can affect the results of the biochemical test, such as the amount of inoculum used, the media used, and the temperature and the length of the incubation period. *S. uberis* utilisation of inulin may be affected by the length of the incubation period (Watts, 1988b).

Misclassification of *S. uberis* has been reported throughout the literature (Kilpper-Balz *et al.*,1982). *Enterococcus faecalis* (*E. faecalis*) has been isolated from cases of mastitis and is closely related to *S. uberis* (Cullen, 1967). *S. uberis* can be confused with *Enterococcus faecalis* (initially named *Streptococcus faecalis*), a catalase-negative, Gram-positive, facultative anaerobe which can grow at both 10°C and 45°C, in 6.5% (w/v) NaCl and at a pH 9.6. *S. uberis* has been reported to tolerate both 10°C and 45°C (Buchanan and Gibbons 1974). Facklam (1977) was able to grow 89% of their *S. uberis* isolates at both 10°C and 45°C. Garvie and Bramley (1979), observed only a slight growth of *S. uberis* II and no growth of *S. uberis* I at 10°C, but no growth of either group at 45°C.

Identification systems:

A multitude of tests and rapid kits have been developed to identify the causative agent of mastitis cases. The majority of the tests on the market for identification of streptococci were designed for isolates from humans. The accuracy of these tests for identification of streptococci of animal origin will depend on the number of veterinary strains incorporated into the database (Watts and Yancey, 1994). Some of the major classification systems for streptococci are the Lancefield precipitin test, phadebact coagulation test kit, Rapid Mastitis Test, Rapid Strep system (Analytical Profile Index 20 Strep), Rapid ID 32, Minitek Gram Positive Set, Strep-Zym, and the Vitrek AMS.

Most complete conventional biochemistry examinations will take at least 5 days (Groothuis *et al.*, 1986). The quicker the identification of the isolates, the better the

test is for diagnostic purposes. Rapid identification systems have been shown to be of some use in the identification of mastitis pathogens (Langlois *et al.*, 1983; Jasper *et al.*, 1985; Rather *et al.*, 1986; Watts and Owens, 1988; Watts, 1989b; Watts and Nickerson, 1986; Watts *et al.*, 1986a; Watts *et al.*, 1986b; Watts and Washburn, 1991).

Lancefield precipitin test

The Lancefield precipitin test was first introduced in 1933 (Lancefield, 1933). The Lancefield precipitin test is used internationally by researchers as the gold standard, when reporting the accuracy of other streptococcal classification tests (Poutrel, 1983, Watts and Owens, 1988). This technique is able to detect at least 95% of streptococcal infections, when compared to the phadebact coagulation test kit (Saxegaard, 1977; Poutrel, 1983). Poutrel (1983) achieved 98% agreement when this technique was compared with the Streptex test kit. The latex agglutination test (also known as the Rapid Mastitis Test) correctly identified 98% of the bovine streptococcal isolates from Lancefield group B, C, D (Poutrel, 1983). One hundred percent of the *S. agalactiae* isolates tested with the latex agglutination test were correctly identified (Watts and Owens, 1988). Some *S. uberis* and *S. dysgalactiae* isolates in the latter trial were classified as *S. agalactiae*.

Co-agglutination test

The co-agglutination test was first used to classify streptococci in 1973 (Christensen *et al.*, 1973). This test correctly identified at least 97% of the bovine streptococcal isolates (Poutrel, 1983; Saxegaard, 1977).

Rapid identification systems

A useful and quick test is the Analytical Profile Index (API) -20 STREP (API Rapid Strep) kit; a 20 well biochemical test kit (Colman and Ball, 1984). A seven digit profile number from the test results is matched with the profile numbers in the index provided from the manufacturer (Facklam, 1984). The accuracy of the API Rapid system depends on the species of isolates being tested, the technique used and the gold standard with which it is compared. The accuracy of the tests to identify streptococcal isolates has been reported to be as low as 39% and high as 100% (Poutrel and Ryniewicz, 1984; Watts, 1989a; Ruoff and Ku, 1983; Groothuis *et al.*, 1986; Watts, 1989b; Jayarao *et al.*, 1991; and Freney *et al.*, 1992). Tillotson (1981) correctly identified 100% of human National Collection of Type Cultures (NCTC) streptococcal isolates, using this technique but could not differentiate between serogroups C and G. The API Rapid system was also unreliable on serogroups C and G as well as S. milleri (Waitkins *et al.*, 1981; Groothuis *et al.*, 1986). Jayarao *et al.* (1991) concluded that this test was less time consuming than conventional biochemical tests and could

identify streptococcal species from the bovine mammary gland with approximately 96% accuracy.

Different results are obtained for different streptococcal species and by different researchers. The API Rapid System can identify the bovine mastitis pathogens *S. agalactiae*, *S. dysgalactiae*, *S. bovis*, *E. faecium*, and *E. faecalis* with 100% accuracy (Tillotson, 1981; Watts, 1989a; Jayarao *et al.*, 1991). Others workers did not obtain the 100% accuracy with some of the aforementioned isolates (Jayarao *et al.*, 1991;Watts and Yancey 1994; Groothuis *et al.*, 1986). Grootnuis *et al.* (1986) concluded that the API Rapid system was useful test for the identification of *S. uberis*. Watts (1989a) identified approximately 96% of the *S. uberis* isolates correctly, using API Rapid Strep. Others had difficulty differentiating between *S. uberis* and *S. bovis* with this technique (Groothuis *et al.*, 1986; Watts and Yancey 1994; Poutrel and Ryniewicz, 1984; Watts, 1989b). Colman and Ball (1984) identified 24 different species of streptococcal isolates from human origin and concluded that the test is useful for identification purposes, but should be used in conjunction with additional biochemical tests.

The API Rapid system gives variable results depending on the length of the incubation period. Groothuis *et al.* (1986) achieved 39% accuracy for the identification of human and animal streptococcal isolates, when the test was incubated for 4 hours, but the accuracy improved to approximately 79% when the same samples were allowed to incubate for 24 hours. Similar results were reported by Watts (1989a) and Jayarao *et al.* (1991). Tillotson (1981) correctly identified 92% of their human streptococcal isolates within only 4 hours, while some authors (Watts 1989a; Jayarao *et al.*, 1991)

reported that *S. uberis* requires a 24 hour incubation period for accurate diagnosis using the API-20. Poutrel and Ryniewicz (1984) identified 75% of their *S. uberis* isolates within the first 4 hours.

The rapid ID 32 Strep test is a kit which contains 32 standardised and miniaturised enzymatic tests. This system requires only a 4 hour incubation for the identification of streptococci. Reading and interpretation of the results can be done automatically or manually using a profile index included in the kit. Fifty-seven organisms from the genus *Streptococcus*, *Enterococcus*, *Aerococcus*, *Erysipelotrix*, *Gardnerella*, *Gemella*, *Lactococcus* and *Listeria* are included in the profile index; but an additional computerised index can be purchased which provides a more comprehensive list of species and which divides *S. uberis* into the two serotypes, I and II.

Some authors have concluded that the profile index is of limited use for identification purposes compared to the table (book) for organism identification and/or the computer database programmes (Freney *et al.*, 1992, Groothuis *et al.*, 1986; Colman and Ball, 1984). Others have noted the misclassification of bacterial isolates of veterinary origin because of the limited database provided in the profile indexes (Watts, 1989b; Jayarao *et al.*, 1991).

Minitek

The Minitek Gram-positive set (MGPS; BBL Microbiology Systems, Cockeysville, Md.) is based on a similar concept to the API kits and uses substrate impregnated disks in 20 wells. Watts (1989b) determined the overall accuracy of the test to be

approximately 35%. It correctly identified 33% of the *S. agalactiae* strains and 74% of the *S. dysgalactiae* strains, while 96% of the *S. uberis* isolates were identified incorrectly as enterococci, because of the positive pyroglutamate (PYR) reaction. All 44 of the tested *S. uberis* isolates were incorrectly classified, 42 were classified as enterococci and one was identified as *S. intermedius* and one as *S. salivarius.* Changing the MGPS database, so that it reads that *S. uberis* to be PYR positive, would change the ability of the MGPS system to identify *S. uberis* isolates correctly to 96% (Watts, 1989b). Approximately 35% of the *E. faecalis* isolates were correctly identified; the others were identified as *E. faecium.* The poor performance of the test was attributed to the limited number of veterinary strains in the database (Watts, 1989b).

Other tests which would require less time than conventional biochemistry for identification of streptococcal isolates are the Strep trio-tube technique and the Vitek Gram-positive system (Vitek AMS). The Strep trio-tube technique correctly identified 98% of the *S. faecalis* isolates within 4 hours (Kim *et al.*, 1987). This technique is recommended for only streptococcal isolates of the Lancefield groups A, B, D, and viridans, not with *S. uberis* isolates which are of the Lancefield E group (Kim *et al.*, 1987). It takes an average of 8 hours to obtain an accurate diagnosis from the Vitek Gram-positive system. The Vitek Gram-positive system correctly identifies approximately 94% of the bovine mammary streptococcal isolates, including 95% of the *S. uberis* isolates (Jayarao *et al.*, 1991).

Phenotypic and genotypic classification:

These non-conventional serological and microbiological techniques are used to describe the phenotypic and genotypic characteristics of mastitis pathogens. Several days are usually required to complete these techniques. The technique used to type the isolate must be able to provide an unambiguous, positive result from each isolate analysed and should be highly reproducible and discriminatory (Maslow and Mulligan, 1993). A test with a high reproducibility is a test from which the same results are obtained on repeated testing of the same strain. If a test has an average reproducibility, approximately 50% of the time the test results are the same on repeated testing of the same isolate. Tests with poor reproducibility will produce different results from repeated testing of the same isolate. A test with a high discriminatory power, will be a test that can be used to accurately differentiate between unrelated strains. A test with an average to poor discriminatory power will correctly differentiate between unrelated strains at best 50% of the time. *Streptococcus spp.* may have common surface antigens which may decrease the discriminatory power of a given test (Poutrel, 1983).

Phenotypic classification

Bacteriophage typing, bacteriocin typing, immunoblotting, and the more commonly used serotyping and antimicrobial sensitivity testing are phenotypic testing techniques, which can be used for the identification of mastitis pathogens. These techniques characterise the physical appearance and behavioural growth characteristics of the organism.

Bacteriophage and bacteriocin typing

Bacteriophage and bacteriocin typing techniques do not discriminate between different species with much accuracy and produce results with limited repeatability (Maslow and Mulligan, 1993). In the bacteriophage typing technique a virus (the bacteriophage) which is capable of infecting and lysing bacterial cells, is utilised for the identification of mastitis pathogens. In bacteriocin typing, toxins produced by other bacteria (e.g. *S. aureus*) are used to determine the susceptibility of the bacteria being tested. Buddle *et al.* (1988) used bacteriocin typing to classify *S. uberis* isolates from several different sites on cow, including milk samples. They determined that the inhibitor profiles performed on repeated cultures remained constant. Out of 39 *S. uberis* isolates cultured, there were 18 different inhibitor profiles, correlating to 18 different strains or types of *S. uberis* isolates. They tested 10 *S. uberis* from the Waikato region and produced 8 different profiles (Buddle *et al.*, 1988). On an individual cow there were different inhibitor profiles from *S. uberis* isolates from the

same site (Buddle *et al.*, 1988). They did not find an association between profiles of isolates found on the teat surface of a cow and on another site on the same animal (Buddle *et al.*, 1988). Tagg and Vulgar (1986) also used this technique to type 15 US strains of *S. uberis* isolates based on their production of and sensitivity to the bacteriocin-like inhibitory substance. Eighty percent of the strains produced the inhibitory substance with nine different production type patterns. The production of the inhibitors varied depending upon the conditions in which the bacteria were grown (Tagg and Vulgar, 1986).

Serotyping and immunoblotting techniques characterise organisms based on their antigenic components. Serotyping detects different antigenic determinants on the cell surface of the same species using immunological techniques. Immunoblotting is an electrophoretic technique that uses antibodies for detection of whole cell or cell surface antigens. This technique is highly reproducible. Serology alone is not enough to classify *S. uberis* taxonomically (Roguinsky, 1972; Stableforth 1959). Roguinsky (1971) had difficulty serologically grouping *S. uberis* because of the cross-reaction with antisera from groups C, D, E, P, and U.

Antibiograms

Determinations of antibiograms for an organism are a common phenotypic characterisation technique. The reproducibility of this technique can be affected by the presence of plasmids (Maslow and Mulligan, 1993).

Antimicrobials:

Preventative measures should be the first line of defence to minimise the incidence of mastitis in a herd. Mastitis prevention programmes, such as the SAMM Plan and the 5-Point Plan, advocate the use of antibiotics at dry off (DCT) and/or during lactation (lactating drugs), in cows "requiring" treatment. The use of the drugs is based on the present and/or historical ICSCC and/or BMSCC and/or a history of clinical mastitis cases. Clinical cases should be treated as they occur (5-Point Plan and SAMM Plan).

Antimicrobials have been used in animals since the early 1940's (Barker, 1945; Benson, 1948; Bryan, 1951). Mastitis is the most common dairy cattle disease treated with antibiotics (Moore and Heider 1984). Initiating the proper antibiotic therapy for the treatment of a mastitis case requires knowledge of the pathogen causing the infection and of the drug(s) to which the pathogen is most sensitive. Culture and sensitivity tests are performed for this purpose. The most commonly used technique for measuring the *in vitro* susceptibility of bacteria to antimicrobial drugs is the agar disc diffusion method. These standardised methods utilise discs laced with the selected antimicrobial concentration, to simulate the achievable antimicrobial serum levels in the patient.

In vitro vs. in vivo tests

If a pathogen is 100% sensitive to a drug *in-vitro*, this does not equate to 100% cure rate when this drug is used *in vivo* against the same pathogen. *In vitro* the organism may be susceptible to different classes of antibiotics, but *in vivo* the bacteria can invade the subepithelial layers and epithelial cells which conceal the pathogen from the action of the circulating antibiotic (Bramley, 1997). Watts and Yancey (1994) noted that the reliability of antimicrobial sensitivity testing was lower in tissue-invading mastitis pathogens. Individual cow factors, pathogen factors, environmental conditions and the pharmacokinetics and pharmacodynamics of the therapeutic agent will affect the response by a cow when treated for mastitis. Antibiotics can alter the neutrophil function (Nickerson *et al.*, 1985); in turn, milk pH and milk components can affect the efficacy of the antibiotic. These factors will result in a discrepancy between *in-vitro* and *in-vivo* drug sensitivities. Milk would be a better medium than non-milk agar mediums for conducting antimicrobial sensitivity testing on mammary pathogens (Owens and Nickerson, 1990; Thornsberry *et al.*, 1997).

Presently the Mastik test (Mastassay) is the only commercially available antibiotic sensitivity test based on veterinary pathogens (Sapersstein, 1993). This is a rapid quantitative antibiotic susceptibility test for mastitis pathogens that are lactose fermenters. The Mastik is a more rapid test than the disc test ((Minimal Inhibitory Concentration (MIC) agar microdilution testing)) and less expensive. The Mastik uses milk-based reagents and the milk sample is used directly without prior bacterial

isolation. Eight antibiotics, at 3-4 concentrations, can be run on one 32 well microtiter test plate. A colour change from purple to yellow after the incubation period is complete, represents a positive response or growth of a lactose fermenter. No colour change represents no growth or the growth of a non-lactose fermenter (pseudomonas, mycoplasma, and Prototheca). The MIC of a lactose fermenter can be determined using this test. The preincubation period with the reagent (sterile milk and bromcresol purple) varies with the type of infection. If the sample is from a clinical case, the preincubation is 1-3 hours, for subclinical and chronic cases 6-8 hours and no preincubation is required for acute cases of mastitis. The activity of an antibiotic is affected by the level of fats, proteins and cations in milk (Watts and Yancey, 1994). Since this is a milk-based test, the results of this test could potentially give more accurate indication of how the drug will react *in vivo*.

The selection pressures on bacteria due to antibiotic use patterns in different veterinary clinics will affect the sensitivity results of a specific pathogen to a particular antibiotic. Resistance can be gained and lost, through the transfer of antibiotic resistance, carried by plasmids, between strains. The overuse and incorrect administration of an antibiotic may result in a higher prevalence of resistance of a particular organism to that antibiotic. Matthews *et al.* (1992) reported that 100% of their 140 bovine mammary streptococcal isolates were sensitive to ampicillin, cephalothin, novobiocin, and penicillin, and 96% of the same isolates were sensitive to tetracyclines. Other authors, reported sensitivities of bovine mammary streptococcal isolates ranging from 60 - 100% for ampicillin, 70-90% for cephalothin, 50 - 90% for erythromycin, and 60 - 100% for penicillins (Hinckey *et al.*, 1985). Matthews *et al.* (1992) reported a low sensitivity of bovine mammary streptococcal isolates to aminoglycosides. Owens *et*

al. noted *S. uberis* isolates were 100% sensitive to penicillin (Owens *et al.*, 1995; Owens, 1997) and a combination of penicillin and novobiocin (Owens, 1997).

Non-antibiotic therapeutics

Use of antibiotic for the control of mastitis can be a common cause of residue violations (Reneau, 1993); therefore, other non-antibiotic alternative therapies are being used to treat mastitis cases. Guterbock *et al.* (1993) noted no difference in clinical or bacteriological cure rates in cases treated with oxytocin vs. amoxyicillin or cephapirin, but the group treated with oxytocin had a higher relapse rate. This study was done in areas in the US where coliform mastitis is the main environmental pathogen; although in this study, ES were the main infections involved. The outcome of this latter study may be different in New Zealand, where ES, in particular *S. uberis*, is the main problem. Researchers in New Zealand are currently reviewing the efficacy of oxytocin as an alternative or adjunct treatment of mastitis in New Zealand herds.

Genotypic classification

The genotypic techniques used to characterise organisms include plasmid profile analysis, restriction endonuclease analysis (REA), southern blot, polymerase chain reaction (PCR), and electrophoretic techniques. These techniques characterise an organism based on the partial or entire genetic constitution of the organism. These techniques are not affected by different culturing conditions or dramatic genetic drift. This makes them more reliable in characterising bacterial isolates.

Plasmid profile analysis

Bacterial plasmids are a self replicating, circular, extrachromosomal DNA molecules, which can be lost or acquired readily by the organism (Maslow and Mulligan, 1993). Plasmid profile analysis is another genotypic technique used for the classification of bacterial pathogens, but is not ideal to use for mastitis organisms because the discriminatory and reproducibility power of the test is less than average.

Southern blot technique

In the southern blot technique, DNA is transferred from an electrophoresis gel to a membrane support (Ausubel *et al.*, 1993). A labelled probe is used to identify the reproduced gel banding pattern on the membrane. The distinctive labelled fragments of DNA in the probe contribute to the technique's excellent power of reproducibility (Maslow and Mulligan, 1993), but its' discriminatory power which is only fair, would limit its' use as a technique for identification of mastitis pathogens, especially for epidemiological studies.

Polymerase chain reaction

The PCR is a technique that may be of use for identification of mastitis pathogens. This genotypic technique, like the southern blot technique, has excellent reproducibility, but the discriminatory power of this technique is average. This

technique is easily contaminated by minuscule amounts of exogenous sequences (Ausubel *et al.*, 1997). Van Belkum and Meis (1994) acknowledge the versatility and the ability of the technique to analyse large numbers of isolates in parallel over a 2 day period.

Electrophoresis

Electrophoresis techniques can be useful for characterising mastitis pathogens. A conventional gel electrophoresis technique consists of the movement of charged particles suspended in a gel under the influence of an electrical current. Conventional electrophoresis lacks discriminatory power for the identification of group B streptococci of human origin (Dohoo and Meek, 1982). Hill and Leigh did note a plasmid, in excess of 40 kb, in a *S. uberis* isolate using the a conventional technique (Erskine *et al.*, 1988). Non-conventional electrophoresis techniques such as contour-clamped homogeneous electric field electrophoresis (CHEF) and the field inversion gel electrophoresis (FIGE) are more discriminatory than the conventional method and have been used on both Gram-negative and Gram-positive organisms of human and animal origin.

Field inversion gel electrophoresis

The FIGE is the most basic and commonly used electrophoretic technique used to type mastitis pathogens. This method utilises a conventional electrophoretic chamber and periodically inverts the orientation of the positive chamber by 180°. Large kb

fragments can be resolved, ranging from 10 - 2000 kb. Lefevre *et al.* concluded that this was not a good technique because the bands obtained from cleaving *S. pneumoniae* using ApaI and SmaI were too big and they obtained different FIGE fingerprints from the same serotype (Berry *et al.*, 1997). This technique was noted to be superior to the CHEF technique for identification of *S. pyogenes* and *S. pneumoniae* from humans (Lefevre *et al.*, 1993; Shah *et al.*, 1994).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was first described by Schwartz and Cantor (1984). They used the technique to study large kilobase (kb) DNA fragments of yeast. Pulsed-field gel electrophoresis techniques are used to resolve DNA molecules >25 kb, because the standard agarose gel electrophoresis has poor resolution above this level (Ausubel *et al.*, 1997). The PFGE is an advanced form of electrophoresis, which periodically inverts the orientation of the positive chamber by variable degrees. The PFGE techniques are useful genotypic techniques for identification and characterisation of mastitis pathogens, because the technique and its' derivations have both excellent discriminatory power and reproducibility (Maslow and Mulligan, 1993; Dohoo and Meek, 1982). PFGE has been shown to be a useful technique for epidemiological studies, of human enterococci (Murray *et al.*, 1990; Prevost, 1992), group B streptococci and methicillin-resistant *S. aureus* and non-resistant *S. aureus* infections (Ichiyama *et al.*, 1991; Wei and Grubb, 1992; Bannerman *et al.*, 1995). Bannerman *et al.* (1995) suggests that this is a useful tool to be used in conjunction with total epidemiological investigational information.

The superior discriminatory power for genotyping bacterial isolates was exhibited in a trial comparing the PFGE to the bacteriophage typing of *S. aureus* isolates of human origin (Bannerman *et al.*, 1995), in which PFGE determined subgroups of the phage groups.

Pulsed-field gel electrophoresis using CHEF is the chosen method for typing *E. faecalis* and *E. faecium* of human origin (Chow *et al.*, 1993; Donabedian *et al.*, 1992; Miranda *et al.*, 1991; Murray *et al.*, 1990; NCCLS, 1990). The CHEF method utilises a specialised electrophoretic chamber with multiple electrodes to apply a highly uniform (homogeneous) electrophoretic field at 120° angles. The CHEF method can resolve bands >2000 kb. Green *et. al.* (1995) used this method on human enterococci and identified bands >500 kb.

The DNA fingerprint pattern of *S. uberis* has been studied by Jayarao *et al.* (1993), who grouped 50 *S. uberis* strains (49 from the US and 1from Great Britain) into 35 different DNA fingerprint patterns. They used Hind III to determine the genomic DNA restriction fragment length polymorphism of the isolates from subclinical and clinical mastitis cases and they grouped the 50 isolates into 8 clusters. The majority of the isolates from Tennessee belonged to one cluster. All the clinical isolates contained DNA fragments $\geq$21kb and most were grouped in 2 clusters. Only 21% of the subclinical isolates contained DNA fragments $\geq$21kb. Identical REA patterns were found only within the same herd. Different clonal types were found within the herds. They concluded from their results that clonal diversity did exist among the *S. uberis* isolates from mastitis cases, and that clonal types could be confined to geographical regions.

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CHAPTER 3

Materials and Methods

The techniques described below were used during the trials reported. Modifications of the procedures are noted in the trials.

Milk sample collection and bacteriological culturing procedure:

Aseptic Milk Collection

Clean the udder/quarters

All excess dirt and mud was brushed and/or washed from the udder, especially around the teat orifice. If udders had to be washed, the available water source in the dairy parlour was used. Only the udders were hosed, making sure not to saturate the underside of the cow, during the washing procedure. The cow was left to drip dry and no manual drying was performed.

Collecting samples from all 4 quarters ("far, near, near, far")

After cleaning the quarters, milk was stripped (two to three initial squirts from the quarters) onto the floor of the shed. The "far, near, near, far" technique was used to prevent contamination of the samples from the collectors' hands and arms. The quarters furthest from the collector were cleaned first. They were wiped with a gauze swab moistened in methylated spirits (approximately 70% alcohol), until the last swab across the tip of the orifice of the teat was clear of debris. The quarters nearest to the collector were wiped in a similar manner. The quarters were then sampled in the opposite direction, sampling the near quarters then the far quarters. The collection tubes were tilted away from the teat at a slight angle (around 45°) with respect to the ventral abdomen to prevent contact of the teat with the opening of the vial. The tilting of the tube also prevented debris from dropping into the sample and contaminating it. A minimum of 15 ml of milk was collected into plastic 30 ml collection vial. The samples were immediately placed in a styrofoam container, for transportation back to the lab. The samples were placed in the refrigerator at 4°C, for no longer than 6 hours before the diagnostic procedures were executed.

Culturing procedure

Single Quarter and Composite Milk Samples

Before the samples were plated onto 5% defibrinated Columbia sheep blood agar (SBA; Fort Richard Laboratories Ltd., Otahuhu, Auckland, New Zealand.) the milk

samples were gently rotated back and forth, to encourage mixing, but prevent the formation of froth. When plating quarter samples or a sample requiring quantitative analysis, a disposable .01 ml loop was used for streaking the sample onto the blood agar plate. For composite samples or those not requiring quantitative analysis, a sterile cotton-tipped applicator saturated with the sample was used for plating. The inoculated SBA plates were placed in an aerobic incubator at 37°C.

Identification of isolates:

The plates were removed from the incubator at 18 - 24 hours and grossly examined for isolated colonies. Characteristic growth was described (size, pigmentation, appearance and haemolysis) and the number of similar colonies was recorded. The plates were placed back into the incubator for an additional 24 hours. At the end 48 hours the colonies were grossly examined and counted again. Gross appearance on blood agar and the biochemical properties of the organisms were used to categorise the organisms into one of eight categories: 1) *Staphylococcus aureus*, 2) *Streptococcus uberis*, 3) coagulase negative staphylococci, 4) *Corynebacterium* spp., 5) *Enterococcus faecalis* 6) gram negative, 7) *Streptococcus agalactiae*, 9) *Streptococcus* spp., or 9) "other" category.

Gross examination

Haemolysis: If there was a zone of haemolysis around the colony, it was initially described as a partial, green, or a clear zone. If the organism was determined to be a *Streptococcus* spp., the zones were described as gamma (δ) haemolysis (no zone of haemolysis), alpha (α) haemolysis (partial zone of clearance) or beta (β) haemolysis (clear zone). If the organism was determined to be a Staphylococcus sp., the initial description of the zones was retained.

Size: The diameter of the colonies was grossly measured. The colonies were described as pinpoint (< 1.0 mm in diameter), small (approximately 1.0 mm in diameter), medium (approximately 2.0 mm in diameter) and large (approximately 3.0 - 4.0 mm in diameter).

Colour and Appearance: The colour of the colonies was noted after the first and second incubation periods. Occasionally the colour of the colonies would change between incubations; therefore, both colours were recorded. The shape and the texture of the colonies were reported as flat, round, moist, raised, creamy, mucoid, and/or dry.

Gram Stain: A commercially available gram stain kit was used to characterise the organism as gram positive (blue to purple on microscopic examination) or gram negative (pink to red on microscopic examination). The microscopic shape of the organisms was reported.

Identification test

Catalase Test: The Catalase Test was used as an indirect measure of somatic cells in a milk sample (Spencer and Simon, 1960). For the purpose of this work, the test was used to detect the production of catalase by a selected organism.

After the gross description, all selected isolates were tested for the presence of catalase. A drop of 3% hydrogen peroxide (H_2O_2) was placed on a glass slide. One typical colony was selected from the SBA plate using a heat sterilised wire loop. The colony was placed in the middle of the drop of H_2O_2. If catalase was present, the test mixture would bubble from the release of oxygen (one of the breakdown products of H_2O_2). The isolates were recorded as catalase positive (bubbles produced) or catalase negative (no bubbles). *Staphylococcus*, *Micrococcus*, *Corynebacterium* spp., coliforms, *Nocardia* spp., *Bacillus* spp. and yeast, are the common milk pathogens that can be catalase positive. *Streptococcus* are catalase negative.

DNase Test: This test detected the presence of DNase in the isolate. This test was performed on catalase positive isolates only. Commercially available DNase plates (Fort Richard Laboratories Ltd., Otahuhu, Auckland, New Zealand) were used. Five samples and one control sample (*Staphylococcus intermedius*) were inoculated per plate using a heat sterilised straight wire. The plates were placed in an aerobic incubator at 37°C, for 24 hours. On completion of the incubation, a thin layer (approximately 3 ml) of 1N hydrogen chloride (HCl) was poured onto the agar containing the samples. The samples were left to stand for approximately 3 minutes, at room temperature. The excess HCl was poured off and the results of the test were

recorded. A positive sample had a clear zone, on both sides of the sample line, measuring at least 3 times the width of the sample line. A negative sample had no clear zone or a clear zone less than 3 times the width of the sample line.

Coagulase Slide Test: This test detects the presence of coagulase production and was performed on catalase positive isolates only. A drop of sterile water, a drop of rabbit serum and 1 - 2 sample colonies (enough to make a milky emulsion) were placed on a glass slide. The mixture was mixed with a heat sterilised loop and the presence of clumping (positive test) was detected. A negative test was one in which no clumping was apparent.

Blood Aesculin and MacConkey Agar (Fort Richard Laboratories Ltd., Otahuhu, Auckland, New Zealand): Only catalase negative isolates were plated. One colony was streaked onto a half plate using a heat sterilised wire loop. The samples on the aesculin plates were aerobically incubated at 37°C for 24 hours. A positive sample hydrolysed the aesculin, darkening the media around the colonies. A negative sample did not hydrolyse the aesculin in the media. MacConkey agar plates, without crystal violet, was used to detect the presence of gram positive lactose fermenters. The isolates streaked on these plates were aerobically incubated at 37°C. The characteristic appearance of the colonies was read and recorded at the end of the 24 and 48 hour incubation period. Colonies that appeared pink to red in colour were denoted as positive and those lacking colour were denoted as negative.

CAMP Test: Only catalase negative isolates were tested. The SBA plates were used. A single CAMP staphylococcal colony was streaked across the centre of a plate and

the test isolates were streaked at 90° angles to this. The plates were aerobically incubated at 37°C for 24 hours. A positive sample had a clear triangular zone of haemolysis around tip of the test strain nearest the staphylococcus.

Definitions

Major Pathogens: Those mammary pathogens, which cause significant changes in the mammary tissue and/or the milk composition. Included in this group of pathogens are *S. aureus*, *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, gram negatives and *Streptococcus* spp.

Minor Pathogens: Those mammary pathogens that cause no to minimal changes in the mammary tissue and/or the milk composition. This group of pathogens are usually commensals of the gland or teat canal and include coagulase negative staphylococci and *Corynebacterium* spp.

Contagious Organisms: Those mammary pathogens whose primary reservoir is the infected udder. The transmission of these pathogens is from cow to cow via the milking equipment or from the milker's hands. The main contagious bacteria are *S. aureus*, *S. agalactiae*, and *Corynebacterium bovis*.

Environmental Organisms: Those mammary pathogens whose primary reservoir is the cow's environment. These organisms are commonly present in the soil, bedding and/or the cow's faeces. The main environmental organisms include *S. dysgalactiae*,

S. uberis, *E. coli*, *E. faecalis*, and *Klebsiella* spp.

Pulsed-Field Gel Electrophoresis

This technique is a modification of two different protocols. The initial design of the procedure was based on the protocol used for the epidemiological examination of *Yersinia* organisms (Fenwick, 1997). *Streptococcus uberis* is structurally different from *Yersinia* spp. *Yersinia* is a gram-negative nonencapsulated bacterium, while S. *uberis* is a gram-positive bacterium that may or may not be encapsulated. When no DNA was extracted from the *S. uberis* isolates using the *Yersinia* protocol, the protocol was modified using steps taken from another PFGE protocol for human streptococcal isolates (personal communication, Martin D, 1996). Adding lysozyme to every lysis step (personal communication, Martin D, 1996) enabled the extraction of the DNA, which was not extracted from the initial attempts.

Day 1: **INITIAL CULTURING**

The majority of the S. *uberis* samples needed to be defrosted (frozen at -80 °C) before being subcultured onto SBA and incubated at 37 °C for 24 - 48 hours (some samples may require 48 hours for optimal growth). A single colony was selected from the latter plates then streaked for isolation on SBA. These plates were incubated at 37 °C for 18 - 24 hours.

Day 2: **INOCULATION**

Three millilitres of brain heart infusion broth (BHIB) was used as the inoculation medium and the cultures from day 1 were used as the inoculum. Four to five colonies were used for samples with colony sizes ≤ 1 mm and 2 - 3 for colony sizes > 1 mm. The inoculated vials were incubated at 37 °C for 16 hours. Both 16 and 24 hour incubation periods produced adequate resolution of bands (Appendix III; figure 1); therefore the shorter incubation time was chosen to decrease the length of time of the procedure.

Day 3: **PLUG PREPARATION and LYSIS**

Step 1: A 200 µl aliquot of each sample (inoculated BHIB) was pipetted into individual sterile eppendorf tube and spun for 5 minutes at 13,000 revolutions per minute (RPM).

Step 2: The supernatant was discarded and the pellet was suspended in 150 µl of ice cold PETT IV (Appendix II) buffer. The samples were spun again for 5 minutes at 13,000 RPM.

Step 3: The supernatant was discarded and the pellet was suspended in 50 µl of ice cold PETT IV buffer.

Step 4: Seventy-five microlitres of molten (60-80 °C) 1% pulse-field certified (PFC) agarose mix (1% pulse-field certified, Bio-Rad Laboratories, Hercules, USA; Appendix II) was added to each isolate.

Step 5: The samples mixed in the agarose were quickly, but carefully dispensed into the plug moulds before they solidified.

Step 6: Sample plugs were formed after at least 10 minutes of solidification on ice.

Step 7: Plugs were placed in individual sterile eppendorf tubes containing 1.0 ml of lysis buffer (Appendix II) overnight (at least 22-24 hours), at 37 °C, in a water bath.

***Day 4*:** **UREA BUFFER**

Step 8: The lysis buffer was decanted and the plugs washed 4 times.

Step 9: One millilitre of ice cold T.E. (pH 8.0) buffer was added to each sample plug. The tubes were gently inverted several times to wash the buffer over the plugs. The samples were left to sit in the buffer for at least 20 minutes. The samples were kept on ice.

Step 10: The solution was decanted and step 9 was repeated 3 additional times.

Step 11: The solution from step 10 was decanted and replaced with 1 ml of the Urea-ESP buffer (Appendix II). The plugs were incubated overnight (at least 22-24 hours), at 56°C, in a water bath.

Day 5: **WASH DAY**

Step 12: The urea buffer was decanted and the plugs washed (see step 9) 4 times (30 minutes between washes) in 1 ml of ice cold T.E. (pH 8.0) buffer).

Step 13: The plugs were left in 1 ml of ice cold T.E. (pH 8.0) buffer and placed in the refrigerator (4 °C) for at least 37 hours before the next step.

Day 6: **ENDONUCLEASE**

Step 14: The samples were removed from the refrigerator and the T.E. (pH 8.0) buffer was decanted. One third of the plug was cut from the original piece and placed in a sterile eppendorf tube. The unused portion was placed back in 1 ml of T.E. (pH 8.0) buffer and placed back in the refrigerator.

Step 15: One hundred microlitres of restriction buffer (Appendix II) was added to the cut slices and left to sit for at least 45 minutes.

Step 16: The restriction buffer was decanted and replaced with 80 µl of cutting buffer (Appendix II). These samples were left to equilibrate on ice for at least 45 minutes.

Step 17: The samples were left in the cutting buffer and left to incubate for at least 4 hours, at 25 °C, in a water bath.

Step 18: After the cutting buffer was decanted the sample plugs were placed in the wells of a 1% PFC agarose gel (Appendix II). A Lambda ladder marker (Bio-Rad Laboratories, Hercules, USA) and the type strain were always in each run.

Step 19: The gels containing the samples were run in the contour clamped homogeneous electric field (CHEF) mapper at a gradient of 6.0 V/cm and a 120 degree angle. The initial and final switch times were 3 and 25 seconds respectively.

Step 20: The gels were removed at the end of the 23 hour run and stained in 1% ethidium bromide for 1 hour.

Step 21: The staining solution was replaced with 800ml of MQ H_2O. The gel was placed in the refrigerator overnight.

Step 22: The gels were illuminated with a UV light (>2500 :W/cm2) and Polaroid photographs were taken.

References

Fenwick SG. Thesis: *Yersinia enterocolitica* infections in people and other animals - a New Zealand study. Massey University, Palmerston North, New Zealand, 1997.

Chapter 4

The influence of two different "intermittent" drying-off strategies on the prevalence of mammary infection in a New Zealand dairy herd

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The influence of two different "intermittent" drying-off strategies on the prevalence of mammary infection in a New Zealand dairy herd

Abstract

Aims. To compare the effect of drying-off techniques on the prevalence of new intramammary infections.

Methods. All lactating cows (n=54) from a single dairy herd were enrolled in the trial. Twenty-seven cows were milked once daily for the last eight days of the lactation (24-hour group) and 27 cows were milked every other day for the same period (48-hour group). There were a total of nine quarter milk sample periods during the trial. Samples 1-3 were taken before the intermittent drying-off procedure began (pre-dry samples). Samples 4 and 5 were taken 2 days prior to dry-off and sample 6 on the day of dry-off. Samples 7-9 were taken within the first 12 days of the subsequent lactation. Bacteriological culture, electrical conductance and rapid mastitis test results of the samples were compared between the two groups.

Results. There was no significant difference of the quarters infected by minor ($p = 0.2661$) or major ($p = 0.4712$) pathogens in the two groups, before the treatments were instituted (pre-dry). Milk production was reduced to 6.3 and 6.6 l/cow/day in the 24-hour and 48-hour, at dry-off. On the last day of lactation (dry-off), the odds of a

quarter in the 48-hour group being infected with pathogens were 2.4 times higher than a quarter in the 24-hour group. These results were confirmed when the random effect of the cow was included (OR = 3.09). There was no significant difference in the prevalence of mastitis at the subsequent lactation in quarters dried-off, during the previous lactation; although, these results may be biased due a loss of a significant number of quarters from the 48-hour group, before the lactation period.

Conclusions. A 48-hour intermittent drying-off technique will increase the prevalence of quarter mastitis caused by minor pathogens, but not major pathogens, at dry-off. There will be no significant difference in the prevalence of mastitis in quarters dried-off with the 24-hour or 48-hour intermittent technique, during the first 12 days of the subsequent lactation.

Key Words. Intermittent, drying-off, bovine, mastitis.

Introduction

Management of dairy cattle for the prevention of mastitis is an important issue in the dairy industry. The current worldwide trend to produce milk of the highest quality compels the dairy industries to continually reassess milk production practices. The Seasonal Approach To Managing Mastitis (SAMM) Plan (Anon, 1995-96) is one approach the New Zealand dairy industry has used to improve milk quality nation-wide, by providing farmers with a suggested management plan to limit the prevalence of mastitis.

The SAMM Plan was developed in the early 1990's by the New Zealand, National Mastitis Advisory Committee. The majority of the suggestions in the Plan are based on the latest research best suited for New Zealand dairying. The SAMM Plan is a guide. It states that cows should be milked once a day for a week prior to the final milking date (Dairy Research Corporation, 1995-96). Intermittent milking before the final day of lactation resulted in a lower incidence of dry period infections, as compared to the "stop" method (milking twice every day up until the last day of lactation) of drying off (Natzke *et al.*, 1974; Oliver *et al.*, 1956a). The ideal interval of "intermittent milking" has yet to be defined. New Zealand dairy farmers practise various intermittent milking intervals before drying off cows.

The purpose of intermittent milking is to accelerate mammary involution and to decrease the cow's milk production before she enters the dry period[1]. In an early study of drying-off techniques, Oliver *et al.* (1956b) suggested there was no overall

1 Busche, T, Oliver, SP, Scannell RE. Changes in milk yield and composition following intermittent or abrupt milk cessation (abstract). Journal of Dairy Science, 67(Suppl. 1), 168, 1984.

difference in the rate of new dry period infections when either the intermittent or the "stop" method of drying-off were used. There was however an increase in infections at dry-off in higher milk yielding cows. Neave *et al.* (1968) noted cows producing greater than 9 litres/day at dry-off developed more new infections than those producing less than 9 litres/day. Oliver *et al.* (1956a) suggested that if cows were uninfected at dry-off, then the "stop" method was more likely to be associated with increased infection incidence compared to an intermittent dry off method. If an infection was already present at dry-off then there were no differences between the methods (Oliver *et al.*, 1956a).

Historically, New Zealand dairy farmers have milked once daily or every other day before dry-off. Newbould and Neave (1965) reported a higher rate of infections in quarters milked once daily as opposed to twice daily before dry-off. A higher rate of infections would be expected if the period between milking was increased prior to the last day of lactation. The purpose of this trial was to compare a 24 and 48-hour milking interval on the incidence of new intramammary infections (IMI), prevalence at the previous dry-off and within the first 12 days of the next lactation.

Materials and Methods

Animals

All remaining lactating Friesian-Holstein cows (n=54) from a single New Zealand seasonal dairy herd were enrolled in the study. Utilising the most recent herd test results (11 days prior to the start of the trial), the 54 cows were listed in descending order by their somatic cell counts (SCC). A coin was tossed to decide which group the first cow on the list would be enrolled in. The second cow on the list went in the group opposite of the first and the third cow on the list went in the same group as the first. This process was continued until all cows were equally divided into the 2 groups.

The cows were managed in the same manner until the start of the intermittent drying-off technique. The cows were split into the two groups and kept in neighbouring paddocks. The only management difference during the trial was the times at which the cows were milked. All milkings were done in the morning. The teats were disinfected with a commercial teat spray after each milking. All cows were being offered silage and pasture above maintenance requirements until 48 hours before the expected dry-off date. All cows were offered only pasture, 48 hours before the last day of lactation. Water intake was not restricted. Individual milk production was measured using a Westfalia (Westfalia Separator NZ Ltd., Auckland) system and recorded on the first day of the trial (pre-dry milk production) and the last day of lactation (dry-off milk production).

Duplicate[2] quarter samples were drawn (samples 1 and 2) from all cows in the trial, for 8 days prior to the dry-off date. Two days later, single quarter samples were drawn (sample 3). Samples 1-3 were the pre-dry samples. All cows were milked intermittently 8 days prior to the dry-off. The cows in the 24-hour group were milked once a day and the cows in the 48-hour group were milked once every other day for 8 days. The 48-hour cows were not brought into the milking shed on days they were not milked. Three samples during the dry-off period were taken from each quarter: 2 days prior to the dry-off date, duplicate quarter samples were taken from all cows (samples 4 and 5); and on the dry-off day a single quarter milk sample was taken from all cows (sample 6). Samples 4-6 were the dry-off samples. Both groups were dried off on the same day. The farmer selected to dry cow treat all four quarters of all cows, based only on the individual SCC results from the herd test taken during the month of their dry-off date. All cows, which had SCC above 150,000 cells/ml, were treated with dry cow therapy (Orbenin Extra Dry, Pfizer Animal Health). Thirteen and 10 cows from the 24 (n = 22) and 48-hour (n = 17) group respectively, that had complete trial data were treated with DCT. All cows were put into one group and managed the same over the dry period and into the lactation period. Cows were not brought back into the milking area during the dry period to be checked for mastitis.

Three samples were taken from each quarter within the first 12 days of the following lactation (lactation samples). Duplicate quarter samples were drawn from all trial cows 7-10 days after calving (samples 7 and 8). A ninth single quarter sample was taken 2 days after the duplicate samples were drawn. All clinical mastitis cases (diagnosed by the farmer and confirmed by bacteriology and physical examination by the primary investigator on the trial) during the trial were treated with intramammary

2 The sampling technique used was part of another study for later publication.

cloxacillin (Orbenin LA, Pfizer Animal Health) at a rate of 1 syringe every 48-hours for 3 treatments. All treatments were recorded.

Samples

About 25 ml of milk was collected aseptically immediately before milking. A cotton gauze moistened in 70% methylated spirits was used to clean the orifice of the quarter before each sample was taken. The samples were promptly placed in a cool styrofoam container, for transportation to the laboratory. The samples were held in the refrigerator at 4 °C for no longer than 6 hours before the diagnostic procedures were completed. All samples were indirectly tested for somatic cell count levels using the RMT and for electrical conductance. Samples were plated onto blood agar plates for bacteriological examination, after the latter tests. The remaining milk samples were held at 4 °C before discard.

Bacteriological examination

A heat-sterilised wire loop was used to transfer 0.01 ml of milk from each sample onto individual blood agar plates. The samples were streaked for isolation and placed into an aerobic incubator for 18-24-hours at 37 °C. Characteristic growth (size, pigmentation, appearance and haemolysis) of the colonies was described. Plates were re-incubated for an additional 24-hours and re-examined. Gross appearance on blood agar and the biochemical properties of the organisms were used to categorise the isolates into one of nine categories: *Staphylococcus aureus, Streptococcus uberis, Streptococcus agalactiae*, Streptococcus spp., *Enterococcus faecalis*, *Corynebacterium*

spp., Coagulase-negative staphylococci, Gram-negative bacteria, or the "mixed minor" (defined below) group.

The following definitions were used:

Minor pathogens: *Corynebacterium* spp., and Coagulase-negative staphylococci.

Mixed minor: More than one type of minor pathogens cultured from a single sample.

Major pathogen: *S. aureus*, *S. uberis*, *S. agalactiae*, *Streptococcus* spp., *E. faecalis*, or Gram-negative bacteria.

Positive culture: When 2 or more colonies (≥200 cells/ml) of the same species were cultured from at least two of the three different milk samples taken from the same quarter over a 3-day period[3].

Negative culture: When fewer than 2 colonies (<200 cells/ml) of the same species were cultured from at least two of the three different milk samples taken from the same quarter over a 3 day period, but two of the three different milk samples had a positive RMT.

Clinical mastitis: Gross abnormal changes in the milk secretions (i.e. clots, changes in consistency) and or physical evidence of udder inflammation (i.e. hard, firm, swollen, haemorrhagic).

3 This will decrease the likelihood of streak canal or transient infections.

Subclinical mastitis: A quarter with a bacterially positive milk culture, but has no gross abnormal changes in the milk secretions or the mammary gland.

Electrical conductivity

A hand held EC meter (Milk Checker, TechniPharm, Rotorua) was used by the primary investigator to determine the electrical conductance of all the milk samples taken during the trial. The investigator did all tests on the samples within two hours after they were drawn from the cows. Approximately 2 mls of milk were poured into the well of the meter to completely cover the electrodes at the bottom of the well. An EC reading equivalent to or greater than 5.5 mS/cm was designated as an infected quarter. Readings less than 5.5 mS/cm were recorded as non-infected quarters.

Rapid mastitis test

For this procedure, about 2 ml of the milk sample and an equal amount of the RMT reagent were added to the well of a paddle. The mixture was gently rotated for about 10 seconds. The amount of precipitation in the mixture was evaluated and scored using a scoring system designed for the California Mastitis Test (Radostits and Blood, 1985) and recorded. A sample was deemed positive if the RMT was 1 or greater. Trace samples were considered negative.

Data analysis

The descriptions of the independent and dependent variables, including the coding of the dichotomous variables used in the data analysis are listed in Table I. The

continuous variables AGE, DLTR (dry-off milk production) and PLTR (pre-dry milk production) were divided into quartiles to create ordinal categorical variables. The 48-hour was the exposed group being compared to the non-exposed (24-hour) group, in the analysis. Univariate and multivariate analyses were performed for the individual quarter results using Pearson's chi-squared analysis and logistic regression. The Mixor (Hedeker, 1993) software was used to perform the logistic regression analysis, using cow as a random effect. The analysis was done with and without the random effect to assess the statistical importance of interdependence of quarters from the same cow with respect to risk of infection.

The values of the odds ratio (OR) and its confidence limits (CL) determined from the analysis were reported. Confidence limits of 90% were used for analysis to increase the chance of finding differences in the risk of exposure, if one existed. It is acknowledged that this will increase the risk of a Type I error. The intra-cluster correlation (ICC) for the dependent variable was reported (Hu *et al.*, 1998). The ICC reflects the interdependence of the quarters within the cow (= clusters) with respect to the variable being tested. An ICC equal to one would represent the status of quarters within a cow being completely dependent on each other.

Table I. The variables used in the univariate and multivariate analysis[a]

Dependent variable	**Description**
SUBCLINICAL MASTITIS	Two groups: 0 = the first group in which no bacteria were cultured from either of the duplicate samples or the bacteria cultured from one of the duplicate samples did not agree with what was cultured from the other duplicate sample, during a specific sample period; 1 = the second group in which the same pathogen was cultured from the duplicate samples taken during a specific sample period.
Independent variables	**Description**
AGE	Four groups: cows 4 and 5 years of age; 6 years; 7 years; and cows ≥ 8 years.
DRY COW THERAPY (DCT)	Two groups: 0 = cows which did not receive DCT at dry-off at the previous lactation; 1 = cows which received DCT at dry-off.
DRY-OFF MILK PRODUCTION (DLTR)	Litres of milk production at dry-off. Four groups: cows producing ≤ 5.55 litres; > 5.55, but ≤ 6.8; > 6.8, but ≤ 7.6; and those producing > 7.6 litres at dry-off.
ELECTRICAL CONDUCTIVITY (EC)	Positive result, if the quarter milk electrical conductivity reading is ≥ 5.5 mS/cm. Negative result, if the quarter milk electrical conductivity reading is < 5.5 mS/cm. The quarter was deemed positive or negative for a given sample period based on the agreement of at least two out of the three samples taken for that period.
PRE-DRY MILK PRODUCTION (PLTR)	Litres of milk production per day before treatments were instituted. Four groups: cows producing ≤ 10.4 litres; >10.4, but ≤ 11.7; > 11.7, but ≤ 13.5; and those producing > 13.5 litres.
QUARTERS (QTR)	Two groups: 0 = the front quarters; 1 = the rear quarters
RAPID MASTITIS TEST (RMT)	Positive result, if the quarter milk RMT reading was 1 and above. Negative result if the quarter milk RMT reading was 0 (negative) or trace. The quarter was deemed positive or negative for a given sample period based on the agreement of at least two out of the three samples taken for that period.
TRIAL GROUP (GRP)	Two treatment groups: 0 = the 24-hour group; 1 = the 48-hour group.

a 0 and 1 are the codes of the dichotomous variables used in the analysis.

Results

Complete data were obtained from 22 and 17 cows from the 24-hour and the 48-hour group respectively. Ten cows from the 48-hour group and 5 from the 24-hour group were not sampled during each of the nine periods. One cow in the 24-hour group with only three functioning quarters, at lactation, was retained in the analysis, because of the small group sizes. Although more cows from the 48-hour group died or were culled; this number was not significant at the cow level ($p = 0.1287$); however, a significant number of infected quarters ($n = 40$) were removed from the 48-hour group compared to the 24-hour group ($p = 0.0001$). These cows were removed from the trial during the period after sample 6 was taken and before sample 7 was taken. The reasons for removal of infected cows in the 48-hour group appeared to be unrelated to the treatments instituted: one cow died within 24-hours after calving, diagnosis not determined, one died from endocarditis, one cow with maternal obstetrical paralysis was humanely killed, and two cows had reproductive diseases unresponsive to therapy. Five cows from the 24-hour and 5 from the 48-hour group were removed from the trial because they were managed differently from the other cows in the trial after sample 6 was taken.

Specific pathogens cultured during the trial period are shown in Table II. The minor pathogens Coagulase-negative staphylococci and *Corynebacterium* spp. were the predominant organism cultured during the all three periods. Relatively low numbers of major pathogens were cultured during this trial. *Staphylococcus aureus* and *Streptococcus uberis* were the predominant major pathogens cultured from quarters in the 24-hour and 48-hour groups respectively. No quarters were infected with *S. agalactiae* or Gram-negative (usually an early lactation cause of mastitis) organisms.

Table II. The number of specific or mixtures of bacteria cultured from the positive cultures during the three periods.

Organism(s)	Before Drying 24-hour (n=108)	48-hour (n=108)	Dry-off 24-hour (n=108)	48-hour (n=108)	Lactation 24-hour (n=87)	48-hour (n=68)
Staphylococcus aureus	3	1	6	2	5	0
Streptococcus uberis	0	4	0	2	3	3
Streptococcus spp.	0	0	0	0	2	1
Enterococcus faecalis	0	0	0	0	2	0
Coagulase-negative *Staphylococcus*	13	24	51	55	20	18
Corynebacterium spp.	25	12	9	9	15	7
Mixed minors	9	3	15	27	9	2

Quarter infection status during the three sampling periods is presented in Tables III & IV. No significant differences were noted in quarters infected by minor pathogens ($p = 0.2661$) or major pathogens ($p = 0.4712$), at pre-dry. There was a significant difference in quarters infected with minor pathogens ($p = 0.0098$), but not major pathogens ($p = 0.5172$), at dry-off. At lactation, the difference in quarters infected with minor and major pathogens was not significant between the two groups, ($p = 0.1778$) and ($p = 0.1082$) respectively. The odds of a quarter being infected with pathogens at dry-off in the 48-hour group were 2.4 times higher than the 24-hour group.

Seven cows (32%) in the 24-hour group and three cows (18%) in the 48-hour group had clinical mastitis in one or more quarters during the lactation sampling period; however, the difference was not significant ($p = 0.2012$). Two of these cows in each of the treatment groups had been treated with DCT, in all four quarters, at dry-off.

Table III. The number of quarters with subclinical mastitis infections caused by minor and major pathogens at before drying, at dry-off, and at lactation

	24-hour group	48-hour group
pre-dry	(n = 108)	(n = 108)
major	3	5
minor	47	39
uninfected	58	64
dry-off	(n = 108)	(n = 108)
major	6	4
minor	75	91
uninfected	27	13
lactation	(n = 87)	(n = 68)
major	12	4
minor	44	27
uninfected	32	37
removed[b]	20	40
major	(0)	(3)
minor	(14)	(35)
uninfected	(6)	(2)

a Only one infection was counted per quarter. If more than one minor or major pathogen was cultured from an individual quarter, than that quarter was considered to be a quarter with a minor or major bacterial infection respectively. If a mixture of major and minor bacterial was cultured from an individual quarter than that quarter was considered to be a quarter with a major bacterial infection.

b Quarters removed from the trial before lactation samples could be taken and the infections they had at dry-off.

Table IV. Number of quarter infections caused by major and minor pathogens, in the two groups, at the different sample periods.

	Minors only		Majors only	
	24-hour	48-hour	24-hour	48-hour
Pre-Dry	n =108	n =108	n =108	n =108
No. infected	47	39	3	5
Odds ratio	0.73		1.70	
90% CL[a]	(0.47 - 1.16)		(0.51 - 5.05)	
Dry-Off	n =108	n =108	n =108	n =108
No. infected	75	91	6	4
Odds ratio	2.36*		0.65	
90% CL[a]	(1.34 - 4.01)		(0.24 - 1.91)	
Lactation	n = 87	n = 68	n = 87	n = 68
No. infected	44	27	12	4
Odds ratio	0.64		0.40	
90% CL[a]	(0.37 – 1.10)		(0.16 - 1.09)	

a CL = confidence limits
* Significant at a 90% CL

Quarter results from both groups were combined for the random effect univariate and multivariate analysis (Tables V and VI). At a confidence level of 90%, the odds of a quarter being infected with pathogens at dry-off in the 48-hour group, was about three times higher than the 24-hour group (Table V, GRP). The estimated relative risk of a quarter having mastitis during the first 12 days of the following lactation, caused by pathogens, in the 48-hour group was about one third less than for quarters in the 24-hour group. The risk of rear quarters being infected with pathogens was at least four and two fold greater than front quarters at dry-off and at lactation respectively. At a confidence level of 90%, there was no increase in the estimated risk of mastitis at the different ages (AGE) and production levels before (PLTR) and after (DLTR) the treatments were instituted, at dry-off or at lactation. The cows in the 24-hour and 48-

hour groups PLTR were on average 11.8 and 11.9 l/cow/d respectively. Their DLTR was on average 6.5 l/cow/d. The risk of quarters with a positive RMT reading being infected with pathogens at dry-off were at least 30 fold greater than for those with negative readings. The risk of quarters with a positive EC reading being infected with pathogens at dry-off were at least two fold greater than for those with negative readings. At lactation, there was not an estimated increase of risk of quarters with positive RMT or EC readings being infected with pathogens compared to quarters with negative readings. The ICC for all independent variables was at least 33%.

Table V. Univariate logistic regression analysis (including random effect): Odds ratios (OR), 90% confidence limits (CL), describing the association between each of the independent variables and quarter mastitis at dry-off and within 12 days in lactation as well as intra-cluster correlations (ICC)

	Subclinical mastitis			
	at dry-off (n = 216)		12 days in lactation (n = 155)	
Independent variable[a]	**OR (90% CL)**	**ICC**	**OR (90% CL)**	**ICC**
GRP	3.09 (1.05-9.08)*	0.344	0.34 (0.13 – 0.93)*	0.367
QTR	4.05 (2.17-7.55)*	0.452	2.15 (1.22-3.80)*	0.415
AGE (years)		0.376		0.378
5	1.00		1.00	
6	0.70 (0.22-2.23)		1.11 (0.18-6.83)	
7	0.70 (0.26-1.89)		[b]	
≥ 8	0.94 (0.62-1.42)		0.76 (0.53-1.10)	
PLTR (litres)		0.370		0.362
≤ 10.4	1.00		1.00	
> 10.4 but ≤ 11.7	0.77 (0.22-2.76)		1.43 (0.43-4.70)	
> 11.7 but ≤ 13.5	1.16 (0.60-2.26)		0.76 (0.36-1.60)	
> 13.5	1.09 (0.56-2.11)		0.68 (0.43-1.08)	
DLTR (litres)		0.334		0.376
≤ 5.55	1.00		1.00	
> 5.55 but ≤ 6.8	0.83 (0.25-2.75)		0.60 (0.16-2.21)	
> 6.8 but ≤ 7.6	2.08 (0.87-4.96)		0.75 (0.37-1.55)	
> 7.6	0.91 (0.60-1.36)		0.66 (0.41-1.04)	
EC	2.44 (1.12-5.31)*	0.336	0.24 (0.05-1.16)	0.402
RMT	30.99 (4.91-195.44)*	0.348	0.62 (0.25-1.27)	0.390
DCT	b	b	1.20 (0.41-3.57)	0.393

*Significant at a 90% confidence level.

a See Table I.

b Insufficient data or not applicable.

The sensitivity (Se), specificity (Sp) and the predictive value of a positive (PVP) and negative (PVN) test for the RMT and EC test are presented in Table VI.

TableVI. The sensitivity (Se), specificity (Sp) and the predictive value of a positive (PVP) and negative (PVN) test for the RMT and EC test at drying off and lactation

	At Dry-Off (%)		At Lactation (%)	
	EC	RMT	EC	RMT
Se	48	39	10	53
Sp	66	97	85	36
PVP	86	98	15	60
PVN	22	26	83	37

The independent variables RMT and QTR were included in the multivariate logistic regression model (Table VII). The relationship between the risk of quarter infection in the two groups and relationship of quarter location was similar to the univariate analysis. The intra-cluster correlation was 61% at dry-off and 39% at lactation.

TableVII. The multivariate logistic regression models (including random effect) for the dependent variable bacterial culture status (SCGP) at drying off and lactation

At Dry-Off	(ICC = 0.611)		At Lactation	(ICC = 0.390)	
Independent Variable	**OR**	**(90% CL)**	**Independent Variable**	**OR**	**(90% CL)**
RMT	32.56	(8.08 – 131.11	QTR	2.23	(1.27 – 3.91)
QTR	2.63	(1.17 – 5.95)			

Discussion

In seasonally milked herds, the majority of the cows in a herd will be dried off on the same day. The SAMM Plan (Anon, 1995-96) provides guidelines for the ideal drying-off technique to use. The type of drying-off technique actually used by the farmer may vary to meet the conditions of the farm and the animals.

Cows are milked intermittently towards the end of the lactation period to decrease milk production. Milk yields are highly associated with the frequency of milking (Wilde and Knight, 1990). The more frequently cows are milked the higher the milk yield; therefore, decreasing the frequency of milking will hasten the involution process (Busche *et al.*, 1984), lowering the milk yield. Some workers have observed that the higher the milk production on the last day of lactation, the greater the risk of intramammary infections during the dry period (Neave *et al.*, 1968; Oliver *et al.*, 1956a). An increase in new infections at dry-off was noted when cows were producing over 9.0 litres/cow/day on the last day of lactation (Neave *et al.*, 1968). In the current study, the average milk production in the two groups prior to treatment was about equal. The cows in the 24-hour and 48-hour groups were producing on average 11.8 and 11.9 l/cow/d respectively. An increase in the incidence of infection was not expected as a result of production at dry-off (Neave *et al.*, 1968), as the cows in this present study were producing on average 6.5 l/cow/d, at dry-off. In this study a decrease in milk production by milking less frequently was not noted because both groups were producing on average about the same amount of milk on the last day of lactation. At a confidence level of 90%, there was no significant increase in the estimated risk of cows developing quarter infections if they were producing greater than 7.6 l/cow/d at dry-off compared to those producing less than 5.5 l/c/d.

Newbould and Neave (1965) reported an increase in infections with decreasing frequency of milking. They reported a higher rate of *S. aureus* infection in quarters milked once every 24-hours as compared to those milked once every 12 hours. This is in agreement with what was observed in the present trial, with regards to overall quarter mastitis infections being higher in the less frequently milked animals. At dry-off, the estimated relative risk of quarter mastitis caused by minor pathogens in the 48-hour group was at least twice as high as quarters in the 24-hour group. A difference in the occurrence of mastitis caused by major pathogens was probably not noted in this trial because the prevalence of major pathogens was low. The prevalence of *S. aureus* and *S. uberis* was less than 6% and 4%, respectively, for all three sample periods. Other *Streptococcus spp.* and *E. faecalis* were only cultured during the lactation period at 2% or less.

Pathogens categorised as major are those, which will cause significant changes in the mammary tissue and/or the milk of the infected quarter, negatively affecting the milk quality. Minor pathogens cause less severe pathological changes compared to the major pathogens; therefore, will have less of an affect on milk quality. Although the prevalence of major pathogens was low and only the increase of mastitis caused by minor pathogens, at dry off, in the 48-hour group was significant, there still may be irreversible changes in the mammary tissue due to even minor changes.

When including the random effect of the individual cows it was noted that the estimated relative risk of quarter mastitis in the 48-hour group tripled compared with the risk of quarter mastitis in the 24-hour group. The random-effect logistic regression analysis (Hedeker, 1993) takes into account the clustering effect of the individual quarters within the cow, which would otherwise be analysed as independent in a fixed-

effects regression model and consequently violate one of the basic assumptions of regression analysis. The importance of adding the random effect of the cow within the analysis was reflected in the ICC. In this analysis the minimum ICC was 33%. Thirty-three percentage and greater ICC reflects a strong degree of interdependence between the quarters of individual cows. The quarters do not act independent of one another. This indicates that any analysis not taking account of this interdependence would result in biased estimates of the regression coefficients.

Although the 48-hour group had a greater number of quarter infections at dry-off, this group had fewer quarters with subclinical mastitis within the first 12 days of the following lactation, compared to the 24-hour group. The odds of a quarter in the 48-hour group having subclinical mastitis was at least one third less than that of quarters having subclinical mastitis in the 24-hour group, with the inclusion of the random effect of the cow. A significant number of infected quarters (40 quarters from 10 cows) in the 48-hour group were removed before the following lactation samples could be taken ($p = 0.0001$). These quarters were removed not because they were infected, but for unrelated diseases or managerial reasons. Compared to the 24-hour group, in the 48-hour group there was at least three times as high (OR = 3.65, 90% CL: 2.05-6.48) an estimated risk of infected quarters being culled before a lactation sample could be taken. Removal of these infected quarters before completion of the lactation period sampling introduced a bias. If there were 100% carry-over of quarter infection status from dry-off to lactation, then there would have been no significant difference in quarter mastitis caused by pathogens, at lactation, between the two groups ($p = 0.9658$). Hillerton and Walton (1991) noted 52% of the infections at dry-off were present during the following lactation. Their data was based on North American dairy herds, but hypothetically applying this to the results of this trial, the difference between

the infection statuses of the two groups would also be non-significant for major and minor ($p = 0.1019$) pathogens.

Mastitis has been correlated with other periparturient diseases, but since necropsies were not done on any of the animals that died or were euthanized during the trial, only an assumption can be made about the demise of the animals from the 48-hour group. Cows with dystocia (Bendixen, 1988), parturient paresis (Curtis *et al.*, 1983, 1985) and retained placenta (Bendixen, 1988; Schukken *et al.*, 1989) had an increase chance of developing mastitis. Maybe the 48-hour treatment increased the susceptibility of the cows to other diseases, by increasing stress and decreasing natural immunity in the cows or possibly providing an increase in systemic pathogens or toxins from the infected mammary gland.

A higher incidence of clinical mastitis has been reported in herds using blanket DCT, as opposed to selective treatment (Macmillan *et al.*, 1983). This was refuted in a later study by Williamson *et al.* (1995), which noted a decreasing incidence of mastitis with blanket DCT. Selective treatment was used in this trial. Cows with individual cow somatic cell counts greater than 150,000 (Dairy Research Corporation, 1995-96) 11 days before the first sample of the trial was taken were treated with a commercial intramammary dry cow product at dry-off. Dry cow therapy has an effect on the outcome of new infections as related to the drying-off method. Natzke *et al.* (1974) concluded that intermittent drying-off is the technique of choice, if DCT is not instituted. If DCT is used, then either the "stop" method or the intermittent method can be used (Natzke *et al.*, 1974). Although a significantly greater number of infected quarters in the 24-hour group was treated with DCT than infected quarters in the 48-hour group ($p = 0.0231$), there was no difference in the estimated risk of quarter

mastitis in the 48-hour group.

The estimated risk of a quarter being infected with pathogens, if the RMT or the EC was positive, was at least 30 and 2 times as likely, respectively, compared to a quarter with a negative reading. These results are in agreement with Charmings *et al.* (1984), who also noted the California Mastitis Test (the RMT is a derivation of this test) and the Fossomatic (A/S Foss Electric, Hillerod, Denmark) were better indicators of subclinical mastitis than the EC meter.

Quarter samples were used as oppose to using combined (composite) samples to increase the Se of the bacterial isolation. The overall prevalence of quarter infections at dry-off was 81% and 56% at lactation.

The Sp of the RMT was greater than 80% during both periods; therefore, the RMT proved to be a reliable indicator of a negative bacterial status of a quarter milk sample at dry-off (Sp = 97%) and lactation (Sp = 83%). The Se of the test was low for both sample periods and the number of false negative test were high. The detection of a positive bacterial status is poor for both periods. If a sample taken during the dry-off period is RMT positive than it is most likely a bacterial positive sample, because the PVP is 98%. The PVP at lactation was only 60%. The RMT is an indirect measure of the number of somatic cells in a tested milk sample. A cow with mastitis will have a higher level of inflammatory cells in her milk than an uninfected cow. As the milk yield declines SCC increases in the milk of both infected and uninfected cows (Lacy-Hulbert *et al.*, 1995). The latter affect is more pronounced when cows are producing less than 5 l/cow/d (Lacy-Hulbert *et al.*, 1995). In the present study only 17% of the cows were producing less than 5 l/cow on the last day of lactation.

The EC meter detects changes in milk composition, particularly the changes in sodium (Na^+), chloride (Cl^-), and potassium (K^+) concentrations (Lacy-Hulbert *et al.*, 1995; Linzell and Peaker, 1971). The electrical conductance of a milk sample is higher from a cow with mastitis than from one without (Chamings *et al.*, 1984; Davis, 1975; Fernando *et al.*, 1982; Greatrix *et al.*, 1968; Linzell and Peaker, 1975). Portable or hand-held meters have been used as a diagnostic tool to detect abnormal milk (Hillerton and Walton, 1991; Okigbo *et al.*, 1984). The Se of the hand-held meter used in this trial was low especially at lactation (10%). The number of false negatives was high, similar to what was determined with the RMT. The probability of detection of a bacterial negative sample was greater at lactation (Sp = 83%) compared to at dry-off (Sp = 66%). Lacy-Hulbert (1995) stated the predictive value of the EC meter is low when the prevalence of infection is low. The PVP at dry-off and lactation was 86% and 53% respectively. An influence of major pathogen infections was not expected in this present trial because the overall prevalence of major infections was low during the trial period (< 10%). Minor pathogens, such as Coagulase-negative staphylococci usually do not cause severe alterations of the mammary parenchyma (Linzell and Peaker, 1971). Ninety-four and 82% of the infections at dry-off and lactation respectively were due to minor pathogens. Contrary to what was expected, a positive result proved to be a reliable indicator of a positive bacterial status of a quarter milk sample, at dry-off, but less reliable at lactation. The PVN for both periods were low; therefore, a negative test result is not a reliable indication of a bacterial negative sample.

The lower production and the higher prevalence of minor pathogens cultured from quarters which were deemed negative on RMT and EC accounts for the high number

of false negatives. The Se, Sp, PVP, and PVN will be affected by the prevalence of the type of bacteria as well as the cut-offs used to signify a sample positive for bacteria. The results here may vary from what others have reported in the literature using these test.

The risk of being infected by pathogens, if the quarter was in the rear was considerably higher than for fore quarters. There was at least a four and two-fold difference (at dry-off and lactation respectively) between the risk of bacterial infection in the rear quarters compared to the fore quarters. The present study supports the work of Cullen and Hebert (1967). They noted significantly higher bacterial counts in the rear quarters after routinely culturing the skin, teat canals and milk of 24 cows, throughout an entire lactation (Cullen and Hebert, 1967). More specifically, Natzke *et al.* (1974) noted that right rear quarters were more often infected than fore quarters. The location and the higher yield of the rear quarters may allow greater exposure to environmental pathogens, increasing the likelihood of mastitis compared to the fore quarters.

Older cows tend to have a higher prevalence of infection (Duitschaever et *al.*, 1972; Reichmuth, 1975; Oliver *et al.* 1956c; Holdaway *et al.*, 1996). The average age of the cows in both groups was 6 years. There was no increased risk of mastitis in older cows compared to the younger cows in the trial. If cows less than 4 years of age were included in this trial a difference may have been noted.

The results from this study have to be interpreted in the context of the sample size and the observed prevalence of specific pathogens. Overall this trial sample size was small, especially at lactation; consequently, a low statistical power was established. About 38% of the cows from the 48-hour group and 19% from the 24-hour group were

prevalence of quarter mastitis caused by minor pathogens at dry-off, compared to using a 24-hour intermittent drying-off technique. In the subsequent lactation, if an assumption of a 52% carry-over of infections from the last day of the previous lactation is made (Hillerton and Walton, 1991), then there will be no significant difference in the prevalence of mastitis in quarters dried-off with the 24-hour or 48-hour intermittent technique.

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Chapter 5

An economic assessment of lactational treatment of subclinical mastitis in individual quarters identified by the rapid mastitis test or the electrical conductivity test

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An economic assessment of lactational treatment of subclinical mastitis in individual quarters identified by the rapid mastitis test or the electrical conductivity test

Abstract

Aims. To assess the economic value of treating subclinical mastitis where the diagnosis is based on electrical conductance and/or the rapid mastitis test results and to determine the cure rates and economic outcome to the dairy farmer.

Methods. One hundred and two Holstein-Friesian dairy cows, from 7 seasonal supply New Zealand dairy herds, were quarter milked sampled twice during early lactation. Quarter samples were taken within 7 days after the first herd test results (pre samples) were received and 14 days post treatment (post samples). The bacteriological culture status, electrical conductance (EC) and the rapid mastitis test (RMT) results were determined on all quarter milk samples. The animals with one or more infected quarters, as determined by EC and/or RMT results, were divided into a treatment or a control group based on their ICSCC at the previous herd test. The groups were further broken down into an uninfected and infected control group and an uninfected and infected treatment group for analysis of the bacteriological culture results. The infected quarters in the treatment group were treated with a course of Spectrazol (Schering - Plough Mallinckrodt Veterinary Limited, Upper Hutt) within 3 days after the initial sample was taken. Pre and post culture, EC, and RMT results were compared between the two groups. The cost of treatment was determined.

Results. The uninfected treatment group cultured significantly more minor pathogens from the pre treatment quarter milk samples than the uninfected control group (X^2 = 8.34, p = 0.0039) and the infected control group cultured more minor pathogens than the infected treatment group (X^2 = 21.67, p = 0.0000). Significantly more major pathogens were cultured from the uninfected control group compared to the uninfected treatment group (X^2 = 8.34, p = 0.0039) and more major pathogens from the infected treatment group compared to the infected control group (X^2 = 21.67, p = 0.0000). Sixty-six percent of quarters infected by major pathogens were diagnosed correctly using electrical conductance and/or the RMT results. Significantly more minor pathogens resolved spontaneously in the infected control group compared to the infected treatment group (X^2 = 5.2, p = 0.0236). The infected treatment group had significantly more *Staphylococcus aureus* infected quarters (X^2 = 5.5, p = 0.0185) on pre sampling compared to the infected control group, but the resolution of the infections was not significant. Individual cow somatic cell counts (ICSCC) decreased in the infected control group and slightly increased in the infected treatment group post treatment.

Conclusion. Treating cows with high somatic cell counts did not significantly lower their cell counts compared to cows left untreated. An assessment of costs and benefits from treating infected quarters, plus the inevitable cost of treatment of uninfected quarters, resulted in an overall net loss of approximately $9.18 per quarter treated.

Key words. Bovine, subclinical mastitis, lactational therapy, economics.

Introduction

Mastitis is one of the most costly diseases to the New Zealand dairy industry. In a herd of 170 cows with a bulk milk somatic cell count (BMSCC) of 400,000 cells/ml mastitis was estimated to cost the producer approximately $14,639 annually (Holdaway, 1992). Approximately 62% of this cost is due to loss of milk production from both subclinical and clinical cases. The greatest monetary loss is due to subclinical mastitis; because for every clinical case observed, 20-30 subclinical cases remain undetected. Clinical cases can be treated with an appropriate antibiotic and the milk diverted from the vat, minimising the chance of penalties due to drug residues and elevated BMSCC. Monthly or bimonthly herd testing can identify subclinical cases, but in the period between testing, undetected subclinical cases can jeopardise milk quality and decrease milk production (Francis, 1982).

In the Seasonal Approach to Managing Mastitis (SAMM) Plan (Anon, 1997) it is suggested, to identify clinical cases of mastitis and treat them appropriately. For subclinical cases, or animals with elevated somatic cell counts (SCC), it is suggested to identify these animals and consider treating cows with SCC above 150,000 cells/ml and heifers above 120,000 cells/ml (Anon, 1997). In an American study, a critical threshold of 500,000 cells/ml was used as a cut-off to decide if a cow had a bacterial infection (Schultz, 1977; NMC, 1990). Dohoo *et al.* (1981) suggested cows with SCC greater than 228,000 cells/ml, with or without clinical signs, were most likely infected with a pathogen. Using a critical SCC threshold set at 250,000 cells/ml, in 3 different New Zealand dairy herds, between 14 - 24% of the quarters were deemed infected (Holdaway *et al.*, 1996). Studies have shown that the critical threshold for minimising

the misclassification of bacterial status of a cow based on SCC varies between individual herds depending on infection prevalence (Sheldrake and Hoare, 1981; Holdaway *et al.*, 1996).

The rapid mastitis test (RMT) and hand held electrical conductivity (EC) meters are two "cow-side" tests, which the farmer can use to further examine a quarter suspected of having mastitis. The RMT is an indirect measure of the SCC, which relies on subjective experience of the user to detect the degree of coagulation of the test mixture. Electrical conductivity meters measure the osmolarity of a milk sample. The greater the degree of damage to the mammary epithelium, the greater the osmolarity of milk, which can be indirectly correlated to health status of the quarter being tested (Davis, 1975; Linzell and Peaker, 1975; Fernando *et al.*, 1985; Hillerton *et al.*, 1991; Milner *et al.*, 1996).

Sheldrake and Hoare (1981) determined a mean sensitivity (Se) and specificity (Sp) of 71% and 81% respectively using a SCC cut-off of 500,000 cells/ml. Sensitivities in the range of 35 - 80% and Sp between 72 - 90% have been obtained in studies using the California Mastitis Test (CMT, similar to the RMT) as a means to correlate the cellular content to the presence of bacteria in milk samples (Barnum and Newbould, 1961; Ewebank, 1962; Johnston *et al.*, 1966). Holdaway *et al.* (1996) determined a Se and Sp of 59% and 56% respectively using an EC meter.

Accurate identification of subclinically infected quarters will influence the value of treatment during the lactation period. Wilson *et al.* (1972) eliminated 60-75% of subclinical infections after treatment during the lactation period, while Mwakipesile *et*

al. (1983) eliminated 63% of infections from quarters. In the latter New Zealand study (Mwakipesile *et al.*, 1983), treated quarters produced 14% more milk after treatment as compared to untreated infected quarters.

The benefit of additional milk production, improved milk quality, and fewer infected animals are factors that may economically outweigh the cost of treatment. The objective of this study was to assess the economic value of treating subclinical mastitis based on the EC and/or RMT results and to determine the cure rates and economic value to the dairy farm.

Materials and Methods

Animals

One hundred and two Holstein-Friesian dairy cows aged 2-10 years, from seven seasonal supply New Zealand dairy herds in the Manawatu and South Taranaki districts, were used in the experiment. All seven herds had a history of periods when BMSCC were elevated above 300,000 cells/ml, during the first few months of the previous lactation. All first lactation cows with an individual cow somatic cell count (ICSCC) $\geq$120,000 cells/ml and all greater than second lactation animals with ICSCC $\geq$150,000 cells/ml were selected from their first herd test of the 1996/97 season.

Individual quarter milk samples were taken from all 4 quarters of the 102 selected cows during both sample periods. The first quarter milk sample (pre sample) was taken within 7 days after the herd test results were received. A quarter was deemed to be infected if one or both of the 2 test results (RMT or EC) were positive (defined below). Two to three days after the initial sample was taken, the selected cows with at least one infected quarter were randomly allocated into one of the two groups, starting with the heifer or the cow with the highest ICSCC. On each individual farm the 2 year olds selected were listed in descending order based on their ICSCC. The older cows were listed in the same manner. A coin was tossed to determine if the first heifer or cow went into the treatment or control group. The second heifer or cow on the list was placed in the opposing group. The third heifer or cow was placed in the same group as the first heifer or cow and the process continued until all animals were allocated to a group. Cows initially selected for the trial, but which had four uninfected quarters

based on the RMT and/or EC quarter milk results were placed in the control group, because no quarter could be selected for treatment. There were a total of 54 cows (216 quarters) in the control group and 48 cows (192 quarters) in the treatment group. The control group consisted of 167 uninfected quarters (uninfected control group – UC) and 49 infected quarters (infected control group – IC). The treatment group consisted of 131 uninfected quarters (uninfected treatment group – UT) and 61 infected quarters (infected treatment group – IT). All infected quarters in the treatment group were treated with cefuroxime (Schering - Plough Spectrazol, Mallinckrodt Veterinary Limited, Upper Hutt), a second-generation cephalosporin, within 3 days after the initial sample was taken. The teat end was cleaned with an alcohol swab, before the antibiotic was infused. The second sample (post sample) was taken 14 days after the last treatment was administrated.

Milk samples

The teats were aseptically prepared before each sample was taken using cotton gauze moistened in 70% methylated spirits. Approximately 25ml of milk was collected from each individual quarter immediately prior to the morning milking. The milk samples were promptly placed in a cool styrofoam container for transportation to the laboratory. The RMT and the EC were done on samples left at room temperature for approximately 1-2 hours. The samples were refrigerated at 4 ^{o}C until bacteriological examination. A composite sample was prepared from approximately 8ml aliquots from each of the individual quarter samples. These composite samples were sent to the Livestock Improvement Centre (Hamilton, New Zealand) for the determination of SCC, using a Fossomatic (A/S Foss Electric, Hillerod, Denmark) machine.

Bacteriological examinations

The bacterial culture results were used as the "gold standard" for deeming the quarters truly infected. Standard procedures for milk culture were performed (NMC, 1990) with the following modifications. A heat-sterilised wire loop was used to transfer 0.01 ml of milk from each sample onto individual sheep blood agar plates. The samples were streaked for isolation and placed into an aerobic incubator for 18-24 hours at 37 °C. The physical characteristics of growth (size, pigmentation, appearance and haemolysis) of the colonies was described. Plates were re-incubated for an additional 24 hours and re-examined. Gram-positive, catalase negative cocci were further differentiated by their ability to hydrolyse aesculin and their ability to haemolyse sheep red blood cells in the presence of a beta toxin produced by alpha-beta staphylococci (CAMP reaction test). Gram-positive, catalase positive cocci were tested for the presence of coagulase. The isolates were categorised into one of seven categories: *Staphylococcus aureus, Streptococcus uberis, Streptococcus agalactiae*, *Streptococcus* spp., *Corynebacterium* spp., Coagulase-negative staphylococcus (CNS), or Gram-negative bacteria. *S. aureus, S. uberis*, *S. agalactiae*, Gram-negative bacteria, and *Streptococcus* spp. were considered major pathogens and *Corynebacterium* spp. and CNS were considered minor pathogens.

EC and RMT

Both tests were performed on samples left sitting at room temperature for 1-2 hours. The primary investigator, a trained veterinarian or veterinary technician performed the tests. A hand held EC meter (Milk Checker, TechniPharm, Rotorua) was used to determine the electrical conductance of all the milk samples. Approximately 2 ml of milk was poured into the well of the meter to completely cover the electrodes at the bottom of the well. Results were recorded. For the RMT, 2 ml of the milk sample and an equal amount of the RMT reagent were added to the well of a paddle. The mixture was gently rotated for about 10 seconds. The amount of precipitation in the mixture was evaluated and scored using a scoring system designed for the California Mastitis Test (Radostits and Blood, 1985) and recorded.

The following definitions were used:

Bacterial cultures ("gold standard")

Positive culture (infected): When 2 or more colonies ($\geq$200 cells/ml) of the same species were cultured from the milk sample.

Negative culture (uninfected): When fewer than 2 colonies (<200 cells/ml) of the same species were cultured from the milk sample.

Electrical conductance (Mas-D-Tec, TechniPharm™, Rotorua)

Base quarter: The quarter milk sample that has the lowest EC reading (also is ≤ 5.5 mS/cm), between the four quarters of a cow.

Infected quarter: A quarter milk sample having an EC measurement of at least 17% greater than the base quarter.

Uninfected quarter: The base quarter and a quarter milk sample which has an EC measurement < 5.5 mS/cm and < 17% over the base quarter.

RMT

Infected quarter: A quarter milk sample with a RMT result of trace or greater (positive).

Uninfected quarter: A quarter with a negative milk RMT result.

Trial groups (quarters)

Infected treatment (IT) group: Infected quarters based on EC and/or RMT results and treated with the chosen intramammary antibiotic.

Uninfected treatment (UT) group: Uninfected quarters based on EC and/or RMT results, but not treated with antibiotics.

Infected control (IC) group: Infected quarters based on EC and/or RMT results, but not treated with antibiotics.

Uninfected control (UC) group: Uninfected quarters based on EC and/or RMT results, but not treated with antibiotics.

Data Analysis

Analysis was performed using Statistix 4.0 (Analytical Software, St. Paul) and Epi Info 6.0 (USD, Incorporated, Stone Mountain), two statistical software programmes. The significance of the change between the initial and final tests was determined using Mantel Haenszel chi-square analysis and Fisher exact when numbers in a group being compared was less than 5.

The Se of the tests was the probability that the diagnostic test correctly identified as positive those quarters being infected with bacteria. The Sp of the tests was the probability that the diagnostic test correctly identified as negative those quarters not infected.

Results and Discussion

Individual quarter SCC were not taken due to the cost, therefore only composite SCC samples (all four quarters from an individual cow combined) were compared. Only the cows from the IC group (n = 28) were compared to the cows in the IT group (n = 47) because there were no cows in the treatment group that did not have at least one infected quarter, to have an UT group, to compare to the UC group. Five cows from the IC group and 1 from the IT was left out of the SCC analysis, because the diagnostic laboratory misplaced one of the SCC results. The average ICSCC for the 2 year olds allocated to the IC and IT groups were approximately 950,000 and 472,000 cells/ml respectively, at the onset of the trial, and 334,000 and 320,000 cells/ml IC group and IT group respectively, at the end of the trial period. The average ICSCC for cows 3 years and older allocated to the IC and IT groups were approximately 1,030,000 and 611,000 cells/ml respectively, at the onset of the trial, and 917,000 and 666,000 cells/ml IC group and IT group respectively, at the end of the trial period. The IC had a higher average ICSCC from the start of the trial because this group was randomly given the 2 year old and the cow 3 years and older with the highest ICSCC. If those two high cows were remove the initial average of the IC group of 2 year olds and cows 3 years and older would be 257,000 and 869,000 cells/ml respectively. The bias of the control having more high ICSCC cows could have been avoided, if all herds were randomly allocated at the same time to the groups, but this could not be done because the cows entered the trial at different times. Listing all the cows in the trial in descending order of ICSCC could have also introduced a bias of herd differences. The groups did show a decline in SCC on the post sample, except the IT group, which

exhibited a small increase in counts. The 2 year old IC group had the greatest decrease in ICSCC. In this trial, treatment of infected quarters, based on EC and RMT results, did not significantly decrease the average ICSCC compared to no treatment of infected quarters.

The comparison of the ICSCC changes were based on the cow within the group and not on her individual quarters. The culture results and analysis were based on the individual quarters within the treatment and control groups. There were 216 quarters (54 cows) and 192 quarters (48 cows) in the control and treatment group respectively. Based on the results of the EC and/or RMT, there were 167 quarters in the control group, which were deemed uninfected (uninfected control group – UC) and 49 quarters deemed infected (infected control group – IC). In the treatment group there were 131 uninfected quarters (uninfected treatment group - UT) and 61 infected quarters (infected treatment group - IT). Only the infected quarters within the treatment group were given antibiotic treatment. The species of bacteria cultured from the individual pre treatment quarter milk samples are displayed in Table I. Bacterial negative cultures constituted the highest percentage of results in both the control and treatment groups. Seventy-five and 33 quarters were positive for bacteria in the UC and IC groups respectively. Seventy-eight and 38 quarters were positive for bacteria in the UT and IT groups respectively. There was a significant difference of minor (including the mixed minor culture results) and major (including the mixed major culture results) pathogens cultured from the pre quarter samples. The UT group cultured significantly more minor pathogens than the UC group ($X^2 = 8.34$, $p = 0.0039$) and the IC cultured more minor pathogens than the IT group ($X^2 = 21.67$, $p = 0.0000$). Significantly more major pathogens were cultured from the UC group compared to the UT group ($X^2 =$

8.34, p = 0.0039). Significantly more major pathogens were cultured from the IT group compared to the IC group (X^2 = 21.67, p = 0.0000). The primary organism cultured from all groups was coagulase-negative *Staphylococcus*, a minor pathogen. *Staphylococcus aureus* was the second most common organism cultured from all groups.

Table I. The number (and percent) of specific bacteria that was cultured from pre treatment quarter milk samples from both the treatment and control groups

Bacteria	Control Group (n=216) uninfected (n=167)	Control Group (n=216) infected (n=49)	Treatment Group (n=192) uninfected (n=131)[a]	Treatment Group (n=192) infected (n=61)[b]
Staphylococcus aureus	24 (14)	2 (4)	10 (8)	10 (16)
Streptococcus uberis	5 (3)	2 (4)	8 (6)	5 (8)
Streptococcus agalactiae	0 (0)	1 (2)	0 (0)	0 (0)
Streptococcus spp.	1 (1)	3 (6)	0 (0)	6 (10)
Gram-negative	2 (1)	3 (6)	4 (3)	1 (2)
Coagulase-negative *Staphylococcus*	36 (22)	20 (41)	49 (37)	11 (18)
Corynebacterium spp.	1 (1)	1 (2)	2 (2)	1 (2)
Mixed minor[c]	1 (1)	1 (2)	3 (2)	1 (2)
Mixed major[d]	5 (3)	0 (0)	0 (0)	3 (5)
Others[e]	0 (0)	0 (0)	2 (2)	0 (0)
No growth[f]	92 (55)	16 (33)	53 (41)	23 (38)

a These are the quarters within the treatment group which were not treated with antibiotics, although another quarter possibly within the same cow was treated with antibiotics
b These are the quarters within the treatment group which were treated with antibiotics
c *Corynebacterium* and CNS cultured from the same milk sample
d Two or more different major pathogens cultured from one milk sample
e Organisms not identified by present bacteriological methods
f No growth after 48 hours of incubation a 37 °C

The post treatment culture results are displayed in Table II. There were 216 and 192 post treatment quarter milk samples taken in the control and treatment group respectively. Approximately equal numbers of minors and major pathogens were cultured from the 4 groups. No significant difference in the number of minor or major pathogens cultured was noted between the groups, regardless to the initial significant difference in the number of major pathogens cultured from the IT group.

Table II. The number of resolved (R) and persistent (P) infections by species of bacteria from pre quarter milk samples and the post sample new intramammary infections (NIMI)

Bacteria	Control Group						Treatment Group					
	uninfected (n=167)			infected (n =49)			uninfected (n=131)[a]			infected (n=61)[b]		
	pre (n=75)[c]		post[d]	pre (n=33)[c]		post[d]	pre (n=78)[c]		post[d]	pre (n = 38)[c]		post[d]
	R	P	NIMI	R	P	NIMI	R	P	NIMI	R	P	NIMI
Staphylococcus aureus	7	17	16	1	1	4	5	5	12	6	4	5
Streptococcus uberis	4	1	12	2	0	1	7	1	5	4	1	5
Streptococcus agalactiae	0	0	1	1	0	0	0	0	1	0	0	1
Streptococcus spp.	1	0	5	2	1	0	0	0	4	5	1	1
Gram-negative	2	0	16	2	1	4	4	0	18	1	0	9
Coagulase-negative *Staphylococcus*	16	20	19	16	4	3	23	26	0	4	7	14
Corynebacterium spp.	1	0	11	0	1	3	2	0	7	1	0	1
Mixed minor[e]	1	0	27	1	0	3	3	0	10	0	1	2
Mixed major[f]	5	0	4	0	0	2	0	0	1	2	1	1
Others[g]	0	0	2	0	0	0	2	0	1	0	0	1
No growth[h]	---	---	16	---	---	21	---	---	40	---	---	6

a These are the quarters within the treatment group which were not treated with antibiotics, although another quarter possibly within the same cow was treated with antibiotics
b These are the quarters within the treatment group which were treated with antibiotics
c These are the results of only the positive pre quarter milk samples
d Bacteria cultured from the post treatment samples include the NIMI and the persistent infections from the pre treatment samples
e *Corynebacterium* and CNS cultured from the same milk sample
f Two or more different major pathogens cultured from one milk sample
g Organisms not identified by present bacteriological methods
h No growth after 48 hours of incubation a 37 °C

If the same species of bacteria was cultured from the pre and post quarter milk sample, the infection was considered to have persisted. If the species of bacteria cultured from the pre treatment sample was absent in the post treatment sample, the infection had resolved spontaneously or from antibiotic therapy. There was no significant difference noted in the resolution of minor and/or major pathogens between the UC and UT groups. There was no significant difference in the resolution of major pathogens in the IC group compared to the IT group. Significantly more minor pathogens resolved spontaneously in the IC group compared to the IT group ($X^2 = 5.2$, $p = 0.0236$).

A high resolution of infections may be a true resolution of the high percentage of teat cistern infections caused by minor pathogens. The initial milk culture may have been

contaminated from bacteria from the streak canal, which may or may not been present on post sampling. When the samples were taken, the milk within the streak canal was discarded to minimise the collection of streak canal contaminants, which may not represent the bacterial infection within the gland cistern. The resolution of infections for both groups would be expected to be much lower with a higher prevalence of major pathogens.

Staphylococcus aureus was the most frequently isolated major pathogen from the pre treatment quarter milk samples. There was no significant difference in the resolution of *S. aureus* infections between the groups, although the IT group, which was treated with antibiotics had significantly more pre treatment *S. aureus* infected quarters than the IC group ($X^2 = 5.5$, $p = 0.0185$). At least 40% of *S. aureus* infections persisted within the groups. Low lactational therapy cure rates for intramammary infections caused by *S. aureus* have been reported (Bramley and Dodd, 1984).

Coagulase-negative S*taphylococcus* were cultured from more than half of the total pre treatment quarter milk samples. This group of organisms has been reported to be approximately 99% sensitive, *in vitro*, to cephalothin, a first generation cephalosporin (McDonald and Anderson, 1981). Swartz *et al.* (1984) suggests CNS are commonly resistant to cephalosporins. Although considered a minor pathogen CNS can be associated with a significant milk loss (Timms and Schultz, 1987).

The accuracy of estimation of the resolution of all the infections may have been reduced by the length of the convalescent period between samples. Although the efficacy of therapeutics for the treatment of mastitis has been based on 7 and 14 days

post treatment culture results (Shpigel *et al.*, 1997), it is suggested that a 3 week convalescent period should be used to account for the possible suppressing affect of the antibiotic (Ziv, unpublished, Beit-Dagan, 1997).

The persistence of specific type of bacteria would best be determined by genomic typing. Although the same species of bacteria was cultured from the quarter on pre and post sampling, it may have been a different type or strain. The bacterial infection may have resolved, but the quarter was reinfected by the same species; therefore, giving the appearance of a persistent bacterium, but it actually had resolved. The actual resolution of bacteria may have been higher than what was reported in this trial.

Table III. The number of specific organisms cultured from the post quarter milk samples that cultured negative on the pre sample

Bacteria	Control Group (n = 108)		Treatment Group (n = 76)	
	uninfected (n = 92)	infected (n = 16)	uninfected (n = 53) [a]	infected (n = 23) [b]
Staphylococcus aureus	2	2	4	1
Streptococcus uberis	9	1	3	1
Streptococcus agalactiae	1	0	1	1
Streptococcus spp.	5	0	3	0
Gram-negative	9	2	9	8
Coagulase-negative *Staphylococcus*	28	1	11	5
Corynebacterium spp.	6	2	3	1
Mixed minor[c]	12	1	2	0
Mixed major[d]	1	0	0	0
Others[e]	1	0	0	0
No growth[f]	18	7	17	6

a These are the quarters within the treatment group which were not treated with antibiotics, although another quarter possibly within the same cow was treated with antibiotics
b These are the quarters within the treatment group which were treated with antibiotics
c *Corynebacterium* and CNS cultured from the same milk sample
d Two or more different major pathogens cultured from one milk sample
e Organisms not identified by present bacteriological methods
f No growth after 48 hours of incubation a 37 °C

Displayed in Table III are the post quarter milk sample results of the pre quarter milk samples that were negative on bacterial culture. Although 80% and 68% of the samples were positive on post sample culturing in the UC and UT group respective,

there was not a significant difference. No significant difference was noted between the IC (56% positive) and IT (74% positive) groups with regards to new infections from quarters negative on pre quarter milk sample.

There were significantly more new intramammary infections (NIMI) caused by major pathogens in the UT group compared to the UC group ($X^2 = 7.34$, $p = 0.0067$), although there was not a significant difference ($p = 0.2111$) noted between the number of contagious major pathogens cultured from quarters in the two groups on pre sampling. The IT group had significantly more NIMI caused by all pathogens compared to the IC group ($X^2 = 6.66$, $p = 0.0099$), although those quarters were treated with antibiotics. This latter difference was not significant between the two groups if major and minor pathogens were looked analysed separately.

The extent of damage to the mammary gland will determine the compositional changes in milk, as measured by EC. The accuracy of detection of infected quarters is dependent on the threshold level chosen to denote an infected quarter. In the present trial, an overall Se and Sp of 21% and 84% respectively was obtained. A high number of bacterial negative samples was diagnosed to be positive (false positives). The ability of the EC meter to detect major or minor infections (Se) was low (Table IV) for both periods. The Sp of the EC was similar for the detection of minor and major pathogens for both the pre and post treatment samples. Chamings *et al.* (1984) identified 91% of the quarters correctly when using a threshold value of 5.8 mS/cm. Holdaway *et al.* (1996) suggested 60% of the quarters would be correctly classified as positive or negative if a threshold level of 6.02 mS/cm was used. Gebre-Egziabher *et al.* (1979) correctly identified 69% of the infected quarters using a conductivity ratio of 1.2.

Table IV. The sensitivity and specificity of the RMT and EC test for samples with positive cultures for major and minor pathogens

Test	Sensitivity (%)	Specificity (%)
RMT1 x major	63	66
RMT1 x minor	38	66
RMT2 x major	55	84
RMT2 x minor	32	84
EC1 x major	33	85
EC1 x minor	15	85
EC2 x major	27	84
EC2 x minor	12	84

1 = pre sample
2 = post sample
major = major pathogens
minor = minor pathogens

The ability to correctly classify a quarter as infected by a major pathogen (Se) using the RMT was 63% for the pre sample and 55% for the post sample (Table IV). The sensitivity of the test declined with the decreasing average ICSCC. The RMT was most effective at correctly classifying bacterially negative samples (Sp) with low somatic cell counts. The overall Se and Sp of the RMT for all samples cultured was 45% and 75% respectively. Similar values for Se in the range of 35 - 80% and for Sp between 72 - 90% have been obtained in other studies using the CMT (Barnum and Newbould, 1961; Ewebank, 1962; Johnston *et al.*, 1966).

The SCC for a milk sample will be affected by the extent of the present and past inflammation in the gland. Bacterial type will affect the level of inflammation in the gland (Schultz, 1977; Erskine, 1992). The majority of the major pathogens cultured were *S. aureus* and the average infected quarter ICSCC in this trial was between 663,000 and 905,000 cells/ml, slightly lower than expected (Schultz, 1977; Erskine, 1992). The RMT detected culture positive quarters when the average ICSCC was at the upper range, more efficiently than the EC; however, the sensitivity was only 63%

for detection of major pathogens and considerably less for detection of minor pathogens.

In this study, BMSCC and ICSCC were used to screen for infected animals. Once the animals were selected, the infected quarters were chosen based on a positive RMT and/or positive EC results. Forty-four percent of the quarters were misclassified as infected. If the 44% of false positive quarters were treated with an intramammary antibiotic 3 times over a 60 hour period plus a 48 hour withholding period applied, as calculated below it would cost approximately $1233.00 in drug costs and discard costs (Table V). This assumes that milk from all 4 quarters will be discarded, as it should be, but does not include the costs of labour, veterinary diagnostics and other veterinary costs. In addition, this does not consider the increased chance of drug resistance and milk antibiotic residue penalties.

Table V. Cost of mistakenly treating 44 "uninfected quarters" during September, due to misclassification, in a 100 cow herd

.89/kg mf[a] x $6.00[b]/kg mf x 3 days x 44 cows[c]	=> $ 705.00
$4.00/tube[b] x 3 x 44 quarters	=> $ 528.00
total	=> $ 1233.00

a The amount of milkfat (mf) in kilograms produced on average by a New Zealand dairy cow during the month of September (Anon, 1995)
b Variable amounts
c Assumes one quarter infected per cow and milk from all four quarters are discarded for 3 days

An economic return from treatment of subclinically infected cows during lactation would arise from elimination of some of the infections and the subsequent increased milk yield from these cured quarters plus the prevention of some new infections in susceptible quarters. Quarters treated successfully with intramammary antibiotics during lactation might produce 14 to 23% more milk then those left untreated (Morris, 1973; Mwakipesile *et al.*, 1983). If the 56 infected quarters were treated and subsequently cured of infection and produced 14% more milk after treatment and were milked from September to the end of May, the negative net return of approximately $9.18/quarter would be obtained (Table VI). This calculation assumes a 100% cure rate. In this trial, only 44% of the treated quarters were cured; therefore the actual cost would be much greater because less milk production would be gained and additional therapy may be needed.

Table VI. Cost and benefit of treating 56 "infected" and 44 "uninfected" quarters during September

1 cow produces 160 kg mf[a]		
40kg mf produced by untreated quarters x .14* quarter	=>	5.6kg more mf produced per treated
5.6kg mf x 56 quarters	=>	314kg mf
314 kg mf x $6.00	=>	$1884
cost of treating 56 quarters (see above calculation)	=>	- $1569
cost of treating 44 uninfected quarters	=>	- $1233 (Table V above)
total net effect	=>	approximately $918 or $9.18/quarter treated

a The amount of milkfat in kilograms produced on average by a New Zealand dairy cow per lactation (Anon, 1995)
b Assumes equal production per quarter and 14% increase in milk yield after successful treatment of all infected quarters

The economics of treating cows with somatic cell counts above 400,000 cells/ml during lactation, with an intramammary drug containing cephapirin as the main active ingredient was previously examined (McDermott, 1983). They achieved a Se of 60% and a Sp of 87% and determined a net loss of $19.75/cow. These investigators did not recommend treating cows during lactation based on somatic cell counts. The cost and benefits calculated in these hypothetical examples will vary depending on many factors including, time of the lactation period, accuracy of detection, type of infection, age of the cow, production potential of the individual cow, duration of antibiotic therapy, cost of the antibiotics used, effectiveness of treatment, and profit from production.

The RMT and EC failed to detect some quarters, which were infected with major pathogenic bacteria; however, these "cow-side" tests provide quick and relatively inexpensive means to identify suspected mastitis quarters when compared to conducting a bacteriological diagnosis. The cost of testing milk samples using an EC meter or the RMT test would include the initial cost of the meter or test and the labour involved for testing. The cost of doing bacteriological diagnosis would also include the cost of labour in addition to the cost of sterile collection vials, alcohol swabs, and laboratory and shipment costs. Aseptic milk sample collection is not required when

performing the RMT or testing for electrical conductance. The labour involved for sampling and/or testing milk samples would be least for determining conductance with a hand held device and greatest for the collection of milk for bacteriological diagnosis because aseptic milk samples need to be drawn. The initial cost of an EC meter, like the one used in this study ranges between $500 and $900. The initial cost of a RMT test kit can be less than $10 to sample approximately 1000 quarters. The average cost of culturing a milk sample in a commercial laboratory is approximately $12 to $20 per milk sample, costing $1200 to $2000 per hundred quarters sampled, which equates to $4800 to $8000 per 100 cows sampled.

The economic assessment in this trial did not attempt to account for all facets involved in the cost of treatment; however, from the variables included in the calculation, a net loss resulted from treatment of cows during the early lactation, when cows were identified by available "cow-side" tests. Although Milner *et al.* (1997) suggested an early detection and effective treatment of mastitis cases would improve milk quality and increase milk yield, the cost may outweigh the benefit. Cows with high ICSCC can not be overlooked, because the presence of high SCC cows in a herd will elevate the bulk milk SCC and reduce milk quality. Treating cows with high ICSCC in this trial did not significantly lower the average ICSCC. If the ultimate goal is to improve milk quality by improving udder health, based on these results, it will be achieved at a net cost to the farmer; therefore the treatment of lactating cows based solely on the reduction of ICSCC is not recommended.

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Chapter 6

Antibiograms of *Streptococcus uberis* isolates from subclinical and clinical cases of bovine mastitis

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Antibiograms of *Streptococcus uberis* isolates from subclinical and clinical cases of bovine mastitis

Abstract

Aims. To determine the antibiotic sensitivity patterns of 150 and 180 *Streptococcus uberis* isolates cultured from subclinical and clinical cases of mastitis respectively, in New Zealand dairy cattle from 15 different regions, using a disk diffusion assay. To assess the suitability of antibiograms for subtyping of *Streptococcus uberis* isolates for epidemiological studies.

Methods. One isolate from the United States and 330 isolates collected from field trials and from five New Zealand diagnostic laboratories were tested for antibiotic sensitivity to the following 12 antibiotics: ampicillin, cephalexin, cephalothin, cloxacillin, erythromycin, gentamicin, lincomycin, neomycin, penicillin G, streptomycin, sulphamethoxazole/trimethoprim, and tetracycline. The isolates were incubated for 18-24 hours at 37°C on Mueller-Hinton agar. The diameter of the zone of inhibition was measured and recorded as sensitive, moderately sensitive (intermediate), or resistant to the antibiotic being tested. Results were compared to a similar study done on isolates from dairy cattle from the United States (McDonald *et al.*, 1976).

Results. The 331 isolates fit into 17 different antibiogram patterns. The subclinical isolates fit into 15 of the 17 patterns and the clinical isolates only 9 of the 17 patterns. Ninety-five percent of the isolates followed one of five common antibiogram patterns.

Eighty percent of the isolates were of antibiogram pattern C, including the single United States of America isolate. Six percent of the isolates followed the pattern D antibiogram and 4% were of the patterns A and I. Less than 1% were of the other 13 patterns determined. No significant difference occurred in antibiograms of clinical compared to subclinical isolates or between isolates of different regions, for all of the antibiotics tested except cloxacillin. The clinical isolates were more sensitive (X^2 = 7.07, p = 0.0078) to cloxacillin compared to the subclinical isolates. Approximately 100% of all the tested isolates were either moderately sensitive or resistant to gentamicin. Similarities in antibiograms between the study isolates and the United States isolates (McDonald *et al.*, 1976) were noted for cloxacillin, cephalothin, erythromycin, lincomycin, penicillin G, and streptomycin. The *Streptococcus uberis* isolates in this study were more resistant to gentamicin (X^2 = 34.51, p = 0.0000) and tetracycline (X^2 = 9.44, p = 0.0021) and more sensitive to neomycin (X^2 = 49.22, p = 0.0000), than the *Streptococcus uberis* isolates from the United States of America (McDonald *et al.*, 1976).

Conclusions. The only significant difference between the subclinical and clinical *Streptococcus uberis* isolates tested in this study was that the clinical isolates were more sensitive to cloxacillin. Sensitivity patterns of *Streptococcus uberis* isolates from different countries may vary with the antibiotic used for testing. The relatively low number of antibiogram patterns resolved from the 331 *Streptococcus uberis* limits the usefulness of the technique as a solitary tool for epidemiological studies.

Key words. *Streptococcus uberis*, antibiograms, dairy cows, mastitis, disk diffusion.

Introduction

Mastitis is one of the most costly diseases occurring in dairy herds. Decreased milk production due to subclinical mastitis, disposal of milk containing antibiotic residues and the cost of veterinary fees and antimicrobial products are costs caused by mastitis. Mastitis treatments constitute the greatest use of antibiotics on dairy farms in the United States of America (Moore and Heider, 1984). Antibiotics are important for the elimination of some bacterial mastitis pathogens that will not spontaneously resolve. Mastitis control programmes should include the use of antimicrobials for treatment of certain cases of mastitis (Anon, 1997). Control programmes should also include culturing and antibiotic sensitivity testing of selected cases to determine what organisms are involved and what antibiotic(s) will best resolve the problems. Using inappropriate or ineffective antimicrobials will be costly resulting in decreased cure rates and an increase in the duration of treatment and milk disposal.

Knowledge of the aetiological agent involved in a mastitis episode is one factor that will effect the efficacy of antimicrobial therapy (Huber, 1977). Carman and Gardner (1997) reported the main mastitis pathogens in New Zealand to be *Staphylococcus aureus* (*S. aureus*), *Streptococcus uberis* (*S. uberis*), *Streptococcus dysgalactiae* (*S. dysgalactiae*) and *Streptococcus agalactiae* (*S. agalactiae*). In their report, *Streptococcus aureus* constituted approximately 40% of the infections and combined infection with *S. uberis* and *S. dysgalactiae* constituted approximately another 40%. Over 80% of isolates cultured from milk samples sent to 5 New Zealand diagnostic laboratories (Animal Health Laboratories in Palmerston North, Lincoln, Invermay, Ruakura and Alpha Scientific in Ruakura), during the 1995 lactation season, were Gram-positive pathogens (personal communication, SR Murray, Mallinckrodt

Veterinary Ltd., Upper Hutt, 1996). Approximately 25% of these samples yielded *S. uberis* isolates. *Streptococcus uberis* is a frequent aetiological agent causing subclinical and clinical mastitis cases in New Zealand dairy herds, during the early lactation and dry periods. The increasing incidence of *S. uberis* mastitis has been reported in New Zealand and other countries. Diernhofer (1930 and 1930a) first described *S. uberis*. If present, these organisms are usually reported in the literature combined with other *Streptococcus* spp. (Schultze and Mercer, 1976; Tyler *et al.*, 1992; Wilson et al., 1996). Streptococci are reported as the most sensitive mastitis bacterial pathogens to antimicrobials (Huber, 1977), although not all *Streptococcus* non-agalactiae pathogens will have the same response to antimicrobial therapies (McDonald *et al.*, 1976; Jousimies-Somer *et al.*, 1996; Owens *et al.*, 1997). Cow factors, clinical status of the infection, strain of the pathogen involved, the antimicrobial used and the route and duration of treatment are some factors affecting cure rates to antimicrobials (Loosemore, 1968; Newbould, 1974; Huber, 1977; Moore and Heider, 1984; Tyler *et al.*, 1992; Craven, 1987; Ziv, 1980 and 1992). It has been shown to be economically beneficial to treat *S. agalactiae* intramammary infections during early or mid-lactation (Yamagata *et al.*, 1987, Erskine and Eberhart, 1990). Cure rates of greater than 90% have been achieved against *S. agalactiae* (Weaver *et al.*, 1986; Prescott and Baggott, 1988; Erskine *et al.*, 1996). Various antibiotics were used to achieve cure rates against *S. uberis* mastitis of between 30 and 100% (Aynsley and Hughes, 1954; Loosemore, 1968; Faull and Ward, 1975; Griffin *et al.*, 1987; Hady *et al.*, 1993; Wilson *et al.*, 1996). In addition to lactational therapy, dry cow therapy has proven efficacious at curing *S. uberis* infections (Clegg *et al.*, 1975; Robinson *et al.*, 1985; Williamson *et al.*, 1995).

The establishment of minimal inhibition concentrations (MIC's) and antibiograms for commonly encountered pathogens in human infections have been reported, especially for some *Streptococcus* species (Tofte *et al.*, 1984; Kim *et al.*, 1987) and *Staphylococcus* species (Hsieh and Liu, 1995; Blanc *et al.*, 1996; Essawi *et al.*, 1998; Na'was *et al.*, 1998) with high levels of resistance to antimicrobials. Some bovine *Streptococcus* species are resistant to antibiotics (Davies, 1961; Matsen and Coghlan, 1972; Nelson, 1969; Toala, 1969). McDonald *et al.* (1976) determined that *S. uberis* isolates cultured from the bovine mammary gland exhibited a higher sensitivity to the 17 antibiotics they tested than *Streptococcus bovis* (*S. bovis*) and enterococci, but less sensitivity than *Streptococcus agalactiae* group G and *Streptococcus dysgalactiae.* Jones *et al.* (1967) reported 8 strains of *S. uberis* to be sensitive to cloxacillin. Pandurango *et al.* (1969) reported 11 cultures of *S. uberis* as sensitive to chloromycetin. Ziv *et al.* (1970) reported differences in the antibiograms of CAMP positive and CAMP negative *S. uberis* isolates, which was contrary to what was reported by McDonald *et al.* (1976). The latter results were not *S. uberis* pathogens from New Zealand dairy cows. Carman and Gardner (1997) reported the antibiotic resistance of 25,000 pathogens cultured from bovine milk samples from New Zealand dairy cows. This was a descriptive study using historical data from the years 1976 to 1995 which reported on five antibiotics.

Antibiograms have been used to subtype isolates from human and animal patients for epidemiological studies (Aarestrup *et al.*, 1995; Blanc *et al.*, 1996). The technique in conjunction with other typing techniques provides a more complete descriptive analysis of isolates being tested (Aarestrup *et al.*, 1995; Hampton *et al.*, 1995; Hsieh and Liu, 1995; Essawi *et al.*, 1998; Na'was *et al.*, 1998; Sloos *et al.*, 1998; Yuan *et al.*, 1998).

The purpose of this study was to phenotypically characterise 331 *S. uberis* isolates cultured from subclinical and clinical cases of bovine mastitis by their antibiotic sensitivity patterns and to determine the usefulness of the technique as a tool for epidemiological investigations. Fifteen regions in New Zealand were represented. The study used 12 antibiotics and results were compared to a similar study performed on *Streptococcus uberis* isolates from the United States of America (McDonald *et al.*, 1976).

Materials and Methods

Streptococcus uberis isolates

The 330 *S. uberis* isolates were cultured from 150 subclinical and 180 clinical cases of bovine mastitis, from 15 regions in New Zealand (Appendix I). The principal investigator collected the subclinical samples and 14% of the clinical samples from field trials. Approximately 86% of the clinical samples came from the five New Zealand Animal Health Laboratories during 1995-7. These *S. uberis* isolates were sent as cultures on blood agar slants, plates or in beef broth. One isolate from the American Type Culture Collection (ATCC) was included in this study for comparison with New Zealand strains. This isolate was sub-cultured and frozen numerous times in a laboratory in New Zealand before it was sent to the principal investigator on sheep blood agar.

Disk diffusion assay

A modification of an alternative method of antimicrobial susceptibility testing (Quinn *et al.*, 1994) was performed. Three to six colonies from a pure culture test sample were selected from a sheep blood agar plate. The colonies were placed in a vial containing 3ml of sterile saline to make a suspension with a turbidity comparable to a 0.5 McFarland turbidity standard. The test suspension was uniformly applied over the entire surface of a 90mm Mueller-Hinton (M-H) agar plate (Fort Richard Laboratories Ltd., Auckland) using a sterile cotton-tip applicator saturated in the test suspension. The inoculated plates were left to stand for 5 to15 minutes before the antimicrobial discs were applied.

The following Oxoid antimicrobial discs (Intermed, Auckland) were used in this study: ampicillin (10 µg), cephalexin (30 µg), cephalothin (30 µg), cloxacillin (5 µg), erythromycin (15 µg), gentamicin (10 µg), lincomycin (2 µg), neomycin (10 µg), penicillin G (10 µg), streptomycin (10 µg), sulphamethoxazole/trimethoprim (25 µg), and tetracycline (30 µg). Three different discs were applied to the surface of the inoculated agar plate using an antimicrobial dispenser (Oxoid, Intermed, Auckland) and a pair of forceps to secure the discs in place. The plates were inverted and placed in an incubator for 18 to 24 hours at 37 °C.

The diameter of the inhibition zone was measured from the back of the M-H agar plate using a metric ruler. The antimicrobial susceptibility was recorded as resistant, moderately sensitive (intermediate), or susceptible based on the diameter of the zone (Table I). These results were compared to the antibiogram results of *S. uberis* isolates collected from dairy herds in the United States of America (McDonald *et al.*, 1976).

Table I: Interpretation of the diameter (mm) of the zone of inhibition (NCCLS, 1990)

Antimicrobial	Diameter of zone of inhibition (mm)		
	Resistant (<)	Moderate Sensitive	Sensitive (>)
ampicillin	21	22-29	30
cephalexin[a]	14	15-17	18
cephalothin	14	15-17	18
cloxacillin[b]	9	10-13	14
erythromycin	13	14-22	23
gentamicin	12	13-14	15
lincomycin[bc]	14	15-20	21
neomycin[b]	12	13-16	17
penicillin G	19	20-27	28
streptomycin	11	12-14	15
sulphamethoxazole/ trimethoprim	10	11-15	16
tetracycline	14	15-18	19

a = a first generation cephalosporin; therefore, used the same inhibition zone differentiation as cephalothin
b = modified from NCCLS, 1983
c = a lincosamide; therefore, used the same inhibition zone differentiation as clindamycin

Data Analysis

Analysis was performed using Statistix 4.0 (Analytical Software, St. Paul), statistical software programme. The number of isolates with an identical antibiogram was determined. The moderately sensitive isolate results were combined with the resistant results for analysis unless specifically stated in the results. The significance of the difference between the subclinical and the clinical isolates from the study and from the United States of American (USA) study was determined using Pearson's chi-square analysis.

Results

The antibiograms for the currently tested *S. uberis* isolates and the *S. uberis* isolates from McDonald *et al.* (1976) are presented in Table II. The 17 antibiogram patterns determined are displayed in Table III.

Table II: The percentage of susceptible New Zealand subclinical and clinical *Streptococcus uberis* isolates to twelve antibiotics compared to other *Streptococcus uberis* isolates (McDonald *et al.*, 1976)

Antimicrobial	Subclinical isolates (n=150)			Clinical isolates (n=180)			McDonald[a] (n=257)		
	S	MS	R	S	MS	R	S	MS	R
ampicillin	99	< 1	0	100	0	0	b	b	b
cephalexin	99	1	0	99	< 1	0	b	b	b
cephalothin	99	< 1	< 1	99	< 1	< 1	100	0	0
cloxacillin	89	10	< 1	97	3	0	95	3	2
erythromycin	97	2	< 1	99	1	0	97	0	3
gentamicin	< 1	67	33	0	48	52	11	2	87
lincomycin	92	7	< 1	96	4	0	95	1	4
neomycin	< 1	30	69	0	11	89	0	1	99
penicillin G	99	< 1	< 1	100	0	0	100	0	0
streptomycin	0	5	95	0	2	98	0	0	100
sulphamethoxazole/ trimethoprim	3	26	71	0	21	79	b	b	b
tetracycline	91	9	0	93	7	0	98	0	2

a = McDonald *et al.* (1976)
b = not tested
S = sensitive
MS = moderate sensitivity or intermediate sensitivity
R = resistant

Table III: The 17 antibiogram patterns of the tested *Streptococcus uberis* isolates (n=331)

PATTERNS	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
ampicillin	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
cephalexin	S	S	S	S	S	R	S	S	S	S	S	S	S	R	R	S	S
cephalothin	S	S	S	S	S	S	S	R	S	S	R	S	S	S	R	S	S
cloxacillin	S	S	S	S	S	R	R	S	R	S	R	R	R	R	S	S	S
erythromycin	S	S	S	S	R	S	S	S	S	S	R	S	R	S	S	R	R
gentamicin	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R
lincomycin	R	S	S	S	R	R	S	S	S	S	R	R	S	S	S	S	S
neomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
penicillin G	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S
streptomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
sulphamethoxazole/ trimethoprim	R	S	R	R	R	R	R	R	R	S	S	R	S	R	R	R	R
tetracycline	S	S	S	R	S	S	R	S	S	S	R	S	S	R	S	S	R
Subclinical (n=150)	8	1	112	9	1	1	2	1	9	1	1	1	1	1	0	1	0
Clinical (n=180)	6	0	153	11	0	0	0	1	5	0	0	1	0	0	1	1	1
USA isolate (n=1)	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	14	1	266	20	1	1	2	2	14	1	1	2	1	1	1	2	1

S = sensitive

R = resistant or moderate sensitivity

Table III: The 17 antibiogram patterns of the 331 tested *Streptococcus uberis* isolates

Conclusion

There was no significant difference between the antibiotic sensitivities in isolates from the subclinical versus the clinical *S. uberis* cases or between isolates from different regions in New Zealand for 11 of the 12 antibiotics, when the moderately sensitive results were combined with the resistant results. The 331 *S. uberis* isolates were divided into17 antibiogram patterns, with 95% of the isolates being in 5 of these patterns. Eighty percent of the isolates exhibited the antibiotic sensitivity pattern C, including the USA sample tested, 6% exhibited pattern D, and 4% patterns A and I. Aarestrup *et al.* (1995) determined 81% of 105 *S. aureus* isolates from bovine milk samples had the same antibiotic pattern. Those isolates (Aarestrup *et al.*, 1995) were 100% sensitive to the 11 antimicrobials tested. The current *S. uberis* isolates with pattern C were sensitive to all antimicrobials tested except gentamicin, neomycin, streptomycin and sulphamethoxazole/trimethoprim. The isolates from subclinical infections were distributed into 15 of the 17 patterns and the clinical isolates only 9 of the patterns. Pattern C was the predominant pattern for both subclinical and clinical isolates.

The isolates from clinical infections were significantly more sensitive to cloxacillin than isolates from subclinical infections ($X^2 = 7.07$, $p = 0.0078$). *Streptococcus uberis* isolated by McDonald *et al.* (1976) had a similar cloxacillin sensitivity pattern to the clinical isolates in this study. Both studies support findings by Jones *et al.* (1967) and Owens *et al.* (1997) of cloxacillin sensitive *S. uberis* isolates. Sensitivities to ampicillin and penicillin G were approximately the same. The testing of both ampicillin and penicillin was redundant since ampicillin is an aminopenicillin with broader activity against Gram-negative aerobes compared to penicillin G, but

approximately the same spectrum of activity against *Streptococcus* spp. Owens *et al.* (1997) reported a 100% sensitivity of *S. uberis* to both ampicillin and penicillin. Cephalexin and cephalothin are both first generation cephalosporins, which are noted to have good activity against many Gram-positive bacteria cultured from the mammary gland (Plumb, 1991). Approximately 99% of the isolates in this study and that of McDonald *et al.* (1976) were sensitive to a first generation cephalosporin. Owens *et al.* (1997) determined that 100% of their *S. uberis* isolates were sensitive to first and third generation cephalosporins. The aminoglycosides (gentamicin, neomycin and streptomycin) have a spectrum of activity primarily against Gram-negative aerobes. Approximately 100% of all the tested isolates were moderately sensitive or resistant to gentamicin, and the clinical isolates had significantly more isolates resistant to gentamicin ($X^2 = 12.94$, $p = 0.0004$). *Streptococcus uberis* isolates were significantly more sensitive to gentamicin ($X^2 = 34.51$, $p = 0.0000$) and tetracycline ($X^2 = 9.44$, $p = 0.0021$) in the study by McDonald *et al.* (1976) when compared to the *S. uberis* isolates in this study. All of the *S. uberis* isolates in the McDonald *et al.* (1976) study were resistant to streptomycin, as well as the current *S. uberis* isolated, when moderately sensitive isolates are considered resistant. Significantly more subclinical *S. uberis* isolates were moderately sensitive to neomycin compared to the clinical isolates ($X^2 = 18.74$, $p = 0.0000$). Significantly ($X^2 = 49.22$, $p = 0.0000$) more isolates were resistant to neomycin in the study by McDonald *et al.* (1976) than isolates obtained in this study.

The antibiograms of *S. uberis* isolates from cases of subclinical and clinical mastitis in New Zealand varied from isolates cultured from the USA. The different management practices and treatment protocols utilised in New Zealand and the USA may contribute to the different antibiogram profiles noted. Similar antibiograms of multiresistant

strains have been cultured from a single source hospital using the same antibiotic regimen over extended periods (Sloos *et al.*, 1998). *Streptococcus uberis* was at least 89%, *in vitro*, sensitive to intramammary antibiotics on the market containing antimicrobials with a spectrum of activity against gram-positive organisms. Less than 3% of the isolates were sensitive to sulphamethoxazole/trimethoprim, gentamicin, and neomycin, *in vitro.* Neomycin is highly nephrotoxic and is combined with other antimicrobials in minute concentrations for intramammary treatment. A combination of antimicrobials may achieve greater cure rates than the individual drugs used alone (Miller, 1977). The sulphamethoxazole/trimethoprim discs were the only combined antibiotics tested in this study.

Disc diffusion methods similar to those used in the present study have been used to determine antibiograms of *Streptococcus* isolates cultured from bovine milk (McDonald *et al.*, 1976; Owens *et al.*, 1997; Thornsberry *et al.*, 1997). The sensitivity of bacterial pathogens to antimicrobials *in vitro* will vary with the type of media used for testing (Aynsley and Hughes, 1954; Louhilehtio *et al.*, 1994; Fang and Pyorala, 1996). Aynsley and Hughes (1954) reported a decreased activity of neomycin in skim milk compared to tryptose broth. Saperstein (1993) reported that an antibiotic sensitivity test that uses a milk medium for testing might have less variation in results when compared to the disc diffusion method.

Placing a control organism along with the test organism on each plate would have increased the reliability of the readings obtained. The antibiogram pattern of the control organism is known; therefore if an incorrect antibiogram pattern for the control organism is obtained during a test, then the test sample results may be inaccurate and the test should be run again. A control was not used in this study due to the increase

cost of additional plates for the study. The same batch of antibiotics disc was used throughout the study to minimise the possible variation in the antibiotic concentration of the disc. The agar plates were obtained from the same source, but this does not exclude the possibility in differences in the concentration of ingredients and thickness of the agar between batches. If the results were repeated after rerunning the test samples that had atypical patterns (the ones with less than 1% of the isolates), this would have increased the reliability of the results obtained, since a control was not used in this study.

The sensitivity of an organism *in vitro* may vary from the *in vivo* response because the environmental conditions for bacterial growth are different (Huber, 1977; Tyler *et al.*, 1992). *Streptococcus uberis* was shown to be sensitive *in vitro* to various tested antimicrobial agents. Although the *in vitro* response may vary from the *in vivo* response, the sensitivity results obtained may be a useful tool in the management of mastitis cases caused by *S. uberis*, because 80% followed a common sensitivity pattern. The antibiogram patterns can be used to eliminate therapies containing antibiotics, which the tested organism was resistant to *in vitro.* If the organism is resistant *in vitro* to a certain antibiotic, it will most likely be resistant *in vivo.* Antibiotic activity is usually decreased in the mammary gland by milk constituents, not enhanced. Many of the intramammary therapies available for use in New Zealand dairy cows contain antibiotics that the tested *S. uberis* were sensitive too, but management of mastitis cases caused by this organism using antibiotics alone will not eliminate the problem, due to the organism's ubiquitous nature in the environment in which cows reside (Cullen, 1966; Bramley *et al.*, 1979; Razavi-Rohani and Bramley, 1981; McDonald, 1984; Smith *et al.*, 1985; Oliver, 1988; Todhunter *et al.*, 1995).

Determination of the environmental sources of the pathogen is necessary to adopt management practices to prevent the spread of the disease to and within a herd.

The current study phenotypically characterised the isolates as being similar isolates, with phenotypic differences primarily noted with cloxacillin. Phenotypic differences did exist between the test isolates and the isolates in the USA study (McDonald *et al.*, 1976). The latter difference may be due to external pressures of the different antibiotic regimes used for the treatment of mastitis in New Zealand vs. the United States.

Antibiogram typing is simple technique to perform (Hsieh and Liu, 1995), but is not very discriminatory (Sloos *et al.*, 1998). Combining this technique with some more discriminatory typing technique, such as ribotyping, biotyping, phage typing, restriction endonuclease analysis and/or other phenotypic and genotypic typing techniques could improve the discriminatory capabilities for subtyping isolates (Aestrup *et al.*, 1995; Hampton *et al.*, 1995; Hsieh and Liu, 1995; Essawi *et al.*, 1998; Na' was *et al.*, 1998; Sloos *et al.*, 1998; Yuan *et al.* 1998) for epidemiological study.

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Chapter 7

Genomic typing of *Streptococcus uberis* isolates from cases of mastitis, in New Zealand dairy cows, using pulsed-field gel electrophoresis

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Genomic typing of *Streptococcus uberis* isolates from cases of mastitis, in New Zealand dairy cows, using pulsed-field gel electrophoresis

Abstract

Aims. To determine and compare the restriction endonuclease analysis pattern of *Streptococcus uberis* isolates from subclinical and clinical cases of mastitis in New Zealand dairy cows, from 15 different farming regions in New Zealand. To compare the pulsed-field genotyping technique with antibiogram typing of *Streptococcus uberis* isolates within 8 different farms.

Methods. The restriction endonuclease analysis pattern of 342 New Zealand and a single United States of America *Streptococcus uberis* isolate was determined using pulsed-field gel electrophoresis. The New Zealand isolates were cultured from milk samples from subclinical and clinical cases of mastitis collected during field research trials and veterinary diagnostic investigations, between May 1996 and January 1998. Each individual pattern of the tested isolates was compared to the pattern of a selected type strain. The degree of dissimilarity of the band patterns between the isolates was compared. These results were compared with the antibiogram patterns defined in Chapter 6, on *Streptococcus uberis* from 8 different farms.

Results. The 343 *Streptococcus uberis* isolates exhibited 330 different restriction endonuclease analysis patterns. The isolate from the United States had a pattern unlike

any of the other isolates tested. Most of the isolates collected were genetically different strains of *Streptococcus uberis*, but we did identify identical patterns within the same quarter of an individual cow, different quarters from the same cow, different cows within the same farm, and from different cows from the same or different districts, farming regions or islands. Seven of the 8 farms had at most only 1 pair of isolates with banding patterns which differed by at most 33% (genetically different isolates), except farm #9, which had 5 pairs of isolates with banding patterns which differed by at most 33%. A high degree of dissimilarity was noted in herds in which all the samples were collected on the same day and in samples collected over a two year period on the same farm.

Fewer antibiogram patterns were defined on each individual farm compared to the number of pulsed-field gel electrophoresis patterns on those farms.

Conclusion. There are at least several hundred genetically different strains of *Streptococcus uberis* isolates in New Zealand capable of causing mastitis in dairy cattle. The high degree of dissimilarity among the isolates tested is an indication that *Streptococcus uberis* infections in New Zealand dairy cattle are largely due to the opportunistic nature of the organism in the cows environment. Prevention and treatment of *Streptococcus uberis* mastitis will therefore need to be directed at a multitude of different strains present throughout the country as well as in individual herds.

The pulsed-field gel electrophoresis typing technique appears to be a more discriminatory test for typing *Streptococcus uberis* isolates on 8 New Zealand dairy

herds than antibiograms.

Key words. PFGE, genomic typing, antibiograms, *Streptococcus uberis*, bovine mastitis, New Zealand.

Introduction

In 1992, the average New Zealand (NZ) dairy farmer spent approximately NZ $14,600 annually to manage and treat mastitis (Holdaway, 1992). Staphylococcal and streptococcal bacteria are the most common aetiological agents involved in subclinical and clinical cases of mastitis in New Zealand dairy cows (Holdaway, 1992). Approximately 81% of the bovine milk samples sent to New Zealand diagnostic laboratories, during the 1995/96 lactation season had positive cultures for Gram-positive bacteria (personal communication, SR Murray, Mallinckrodt Veterinary Ltd., Upper Hutt, 1996). A high percentage of these samples were positive for *Staphylococcus aureus* (37%) or *Streptococcus uberis* (31%). The use of dry cow therapy (DCT) and post-milking teat disinfectants are reducing the prevalence of contagious organisms, like *Staphylococcus aureus* (*S. aureus*), while mastitis caused by environmental pathogens such as *Streptococcus uberis*, are becoming increasingly common (Oliver and Mitchell, 1984; Smith *et al.*, 1985; Oliver, 1988; Todhunter, 1995).

Mastitis caused by *Streptococcus uberis* (*S. uberis*) appears to be increasing in dairy cattle populations internationally (Cullen, 1969; King, 1981; Bramley and Dodd, 1984; Oliver, 1988; Watts, 1988; Oliver *et al.*, 1990). *Streptococcus uberis* is an important environmental pathogen involved in cases of subclinical and clinical mastitis during the early lactating and nonlactating periods (Hogan and Smith, 1997; Oliver *et al.*, 1997). In particular, *S. uberis* accounted for approximately 90% of the 12.2% total cases of environmental streptococcal mastitis, in heifers, within the first 5 days of lactation (Pankey et al., 1996).

Prevention and control of *S. uberis* is important to minimise the monetary losses due to environmental pathogens. *Streptococcus uberis* has a ubiquitous and widespread distribution in the cow's environment (Cullen, 1966; Newbould, 1975; Bramley, 1978 and 1979; Smith *et al.*, 1985), as well as occurring in extramammary and intramammary sites on the cow. This may contribute to the poor ability to control *S. uberis* mastitis with post-milking teat disinfectants and DCT (Pankey *et al.*, 1982; Oliver and Sordillo, 1988; Oliver *et al.*, 1989; Hillerton *et al.*, 1995; Williamson *et al.*, 1995). Vaccines directed toward environmental pathogens can decrease the proportion of and severity of clinical disease (Hogan et al. 1992a and 1992b). If cows are vaccinated against a strain of bacteria, and become subsequently infected with that strain they exhibit higher milk yields, lower somatic cell counts (SCC) and shed fewer organisms compared to the unvaccinated cows (Leigh, 1994b).

Variable responses of an animal to a particular vaccine will be dependent upon the strain of bacteria the animal is subsequently infected with (Slanetz, 1963; McDowell, 1974). The efficacy of a vaccine against *S. uberis* mastitis in New Zealand dairy cattle would need to be studied using strains infecting dairy cows in New Zealand. Buddle *et al.* (1988) typed 39 *S. uberis* isolates from extramammary and intramammary sites on dairy cows using the bacteriocin inhibition typing technique. They obtained 18 different production type patterns from the 39 isolates. Ten of the isolates they typed from the Waikato Region produced 8 different profiles. Isolates with different profiles were present from the same site on the same cow. These results suggest *S. uberis* strains in certain farming regions in New Zealand may be a heterogeneous group of organisms.

The accuracy of the bacteriocin inhibition typing technique to discriminate between different strains of organisms is poor compared to the pulsed-field gel electrophoresis (PFGE) technique, which has an excellent ability to discriminate between similar and dissimilar strains (Dohoo and Meek, 1982; Maslow and Mulligan, 1993). The PFGE genomic classification technique is useful for subtyping Gram-negative (Allardet-Servent *et al.*, 1989; Arbeit *et al.*, 1990; Mogollon *et al.*, 1990; Murray *et al.*, 1990; Grouthues and Tummer, 1991; Yan *et al.*, 1991) and Gram-positive bacteria (Skjold *et al.*, 1987; Hogan *et al.*, 1988; Goering, 1990; Ichiyama *et al.*, 1991; Miranda *et al.*, 1991; Poddar and McClelland, 1992; Prevost *et al.*, 1992; Wei and Grubb, 1992; Saulnier, 1993; Bannerman *et al.*, 1995; Hermans *et al.*, 1995) from human or animal sources. The production of restriction endonuclease analysis (REA) patterns using conventional agarose gel electrophoresis (Hill and Leigh, 1989; Jayarao *et al.*, 1991, 1992, and 1993) or recently PFGE (Baseggio *et al.*, 1997) are useful tools for subtyping *S. uberis* isolates. Jayarao *et al.*, (1991) collected 42 *S. uberis* isolates from milk samples from 17 cows and produced 17 REA patterns. They determined that paired isolates from individual cows could have similar or dissimilar REA patterns. They isolated similar strains from different quarters in the same cow on the same day of collection and at different collection dates. They confirmed persistent infection through the isolation of similar strains from the same quarter on different collection dates. Dissimilar strains were isolated from the same cow but from different quarters and from the same quarter on different collection times. Jayarao *et al.*, (1992) grouped 22 strains, from 9 cows, of *S. uberis* isolates from cases of subclinical and clinical mastitis, into 12 REA patterns. Baseggio *et al.* (1997) noted of the three common streptococcal bacteria involved in bovine mastitis (*Streptococcus dysgalactiae*,

Streptococcus agalactiae, and *Streptococcus uberis*), *S. uberis* was the most heterogeneous of the group. They subtyped 21 isolates from 10 herds using a PFGE technique and only one herd had 2 isolates with identical REA patterns. Another herd had two isolates in which patterns differed by one band, but these were considered to be clonally related (Baseggio *et al.*, 1997).

Genomic typing of *S. uberis* was done on a limited number of samples from localised regions in the United States of America (Jayarao *et al.*, 1991 and 1992) and Australia (Baseggio *et al.*, 1997). With New Zealand being a smaller and more isolated country, *S. uberis* strains involved in bovine mastitis cases may be a more homogeneous group. The objective of this trial was to determine and compare the REA patterns of 342 *S. uberis* isolates from subclinical and clinical cases of mastitis in dairy cows, from 15 different farming regions in New Zealand.

Materials and Methods

Streptococcus uberis isolates

The 161 *S. uberis* isolates from subclinical cases of mastitis were isolated from single or duplicate quarter milk samples, collected from Holstein-Friesian lactating cows from farms in 5 farming regions in New Zealand (Table I). The milk samples were collected for research purposes or for routine veterinary diagnostic procedures, between May 1996 and January 1998. The districts and farming regions from which the 181 clinical samples were collected are listed in Table II. Twenty-five of the *S. uberis* isolates were collected for veterinary diagnostic purposes by the chief investigator and 156 of the isolates came from the five New Zealand Ministry of Agriculture and Forestry (MAF) Veterinary Diagnostic Laboratories (Auckland Animal Health Laboratory, Invermay Animal Health Laboratory, Lincoln Animal Health Laboratory, Palmerston North Animal Health Laboratory, and Ruakura Animal Health Laboratory). All the isolates were profiled as *S. uberis* I or II by the Rapid ID 32 Strep Kit (bioMerieux Vitek, Inc. Hazelwood, Missouri). Isolates were stored in 1 ml of glycerol broth at –80°C, until thawed for testing purposes.

Table I: The number (#) of subclinical samples from districts and New Zealand Farming Regions

Districts	*New Zealand Farming Region**	*# Samples*
Whangarei	Northland	3
Franklin	Central Auckland	2
Matamata	South Auckland	4
South Taranaki	Taranaki	20
Manawatu	Wellington	95
Horowhenua	Wellington	37
	TOTAL	**161**

* see Appendix I; figure 1 and 3

Table II: The number (#) of clinical samples from districts and New Zealand Farming Regions

Districts	*New Zealand Farming Region**	*Island*	*# Samples*
Far North	Northland	North	2
Waipa	South Auckland	North	9
Hauraki	South Auckland	North	3
Matamata	South Auckland	North	15
Waikato	South Auckland	North	7
Tauranga	Bay of Plenty	North	6
Whakatane	Bay of Plenty	North	6
Western Bay of Plenty	Bay of Plenty	North	2
Waitomo	Western Uplands	North	1
Rotorua	Central Plateau	North	9
Gisborne	East Coast	North	1
New Plymouth	Taranaki	North	4
South Waikato	South Waikato	North	7
South Taranaki	Taranaki	North	18
Rangitikei	Wellington	North	3
Central Hawkes Bay	Hawkes Bay	North	3
Tararua	Wairarapa	North	2
Carterton	Wairarapa	North	1
South Wairarapa	South Wairarapa	North	6
Manawatu	Wellington	North	36
Horowhenua	Wellington	North	3
Upper Hutt	Wellington	North	1
Kaikoura	Nelson/Malborough	South	2
Hurunui	North Canterbury	South	3
Dunedin	Otago	South	2
Clutha	Otago	South	4
South Otago	Otago	South	19
Gore	Southland	South	5
Southland	Southland	South	1
TOTAL			**181**

* see Appendix I; figure 1 and 4

Preparation of genomic DNA

The preparation of the agarose blocks was a modification of earlier procedures performed on Gram-positive and Gram-negative bacteria (personal communication, Martin D, 1996; Fenwick, 1997). A 3 ml vial of Brain Heart Infusion Broth (BHIB) was inoculated with one to five colonies (dependent on the size) of a 24 to 48 hour old culture of the sample strain. The inoculated broth was aerobically incubated at 37 °C for 16 hours. The vials were rotated gently to suspend the cellular debris at the bottom of the vial into solution. Two hundred microlitres of the broth culture were pipetted into a sterile micro-centrifuge tube and centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and the sedimented cells re-suspended in 150 µl of ice-cold Pett IV buffer (1 M NaCl, 10 mM Tris HCl pH 8.0, 10 mM di-Na^+ EDTA pH 8.0). The sedimented cells were re-suspended in 50 µl of ice-cold Pett IV buffer before 75 µl of molten 1% pulse-field certified agarose (PFC-Bio-Rad; 10 mg ml^{-1} in Pett IV buffer) gel was added. The suspension was carefully loaded into the plug moulds and left to solidify on ice for at least 10 minutes. Once solidified the plug was incubated in 1 ml of lysis buffer (1 M NaCl, 10 mM Tris HCl pH 8.0, 100 mM di-Na^+ EDTA pH 8.0, 0.5% Sarkosyl, 0.2% Na-deoxycholate, 1 mg ml^{-1}) for 22-24 hours in a water bath at 37°C. The lysis buffer was decanted and the plug washed 4 times with ice-cold TE pH 8.0 buffer (10 mM Tris HCl pH 8.0, 1 mM di-Na^+ EDTA pH 8.0) for 20 minutes. The plug was then placed in 1 ml of Urea-ESP buffer (6 M Urea, 50 mM Tris HCl pH 8.0, 50 mM di-Na^+ EDTA pH 8.0, 1% sodium lauryl sarcosine, 0.2% sodium deoxycholate, 0.5 mg ml^{-1} Proteinase K) for 22-24 hours in a water bath at 56°C. The Urea-ESP buffer was decanted and the plug washed 4 times with ice-cold TE pH 8.0 buffer for 30 minutes. The plugs were stored in 1 ml of ice cold TE pH 8.0 buffer in a

refrigerator at 4 °C.

Digestion of genomic DNA with Sma I

The genomic DNA in the agarose plugs was left to mature for at least 37 hours before digestion. Before digestion, one third of the initial plug was equilibrated on ice in 100 µl of restriction buffer (12 µl 10 x New England Biolabs Buffer 4, 10 mg ml^{-1} of Bovine Serum Albumin) for at least 45 minutes. The restriction buffer was decanted and the plug was placed in 80 µl of cutting buffer (8 µl 10 x Buffer 4, 10 mg ml^{-1} of Bovine Serum Albumin, 15 U of Sma I) on ice for at least 45 minutes before incubation overnight in a water bath at 25 °C.

Pulsed-field gel electrophoresis

The DNA fragments of the test samples and one or two markers (Bio-Rad, DNA size standards-Lambda Ladder, catalogue # 170-3635) were electrophoresed through a 1% PFC agarose gel (800 mg in 0.5 x TBE buffer – 1 M TRIS base, 1 M HBO_3, 2 mM di-Na^+ EDTA pH 8.0) using a contour-clamped homogeneous electric field (CHEF) apparatus. The gel was set to run for 23 hours, at 6 V cm^{-1}, pulse angle of 120°, and buffer temperature of 14 °C. Two litres of buffer (1.8 ml of sterile distilled water and 200 µl of the 5.0 x TBE buffer) circulated at 1 L min^{-1}. The initial and final switch times were 3 and 25 seconds respectively. Fifteen to 18 plugs, including the type strain #427 (reference strain) and marker (Bio-Rad Laboratories, Hercules, California) were run with each assay.

Staining and photographing the gel

On completion of the assay, the gel was stained in 1% ethidium bromide (80 µl of 10 mg ml^{-1} solution in 800 ml of sterile distilled water) for an hour, then de-stained in 1L of sterile distilled water and set in a refrigerator overnight at 4°C. The gels were illuminated with an UV light (>2500:W/cm^2) and Polaroid photographs (Fuji FP-3000B black & white film) were taken.

Interpretation of genomic DNA fragmentation pattern

The photographic image of each gel was scanned and the graphic image enlarged for ease of visual interpretation. Strain #427 was arbitrarily designated as type strain A and run on every gel along with a marker. For analysis, all other tested strains were compared to the reference type strain A. A template of the banding pattern of type A, for that particular gel, was drawn on a transparent sheet. The spatial relationships of the bands of type A were repeatable across the different gels. The locations of the bands of the tested strains and the markers were compared to the template. The strains were analysed from the higher molecular weight bands to the lowest. The presence of a band in the test strain for a particular location was noted as a "1" and a "0" was given for the absence of a band for the given 58 band locations. The analysis of the degree of dissimilarity of patterns of bands between isolates was performed using the cluster analysis procedure in the statistical software NCSS 97 (Number Cruncher Statistical Systems, Kaysville, Utah). The cluster analysis algorithm was based on the linkage type group-average for unweighted pairs and the Euclidean distance method. Isolates with identical banding patterns would have a degree of dissimilarity equal to zero. If

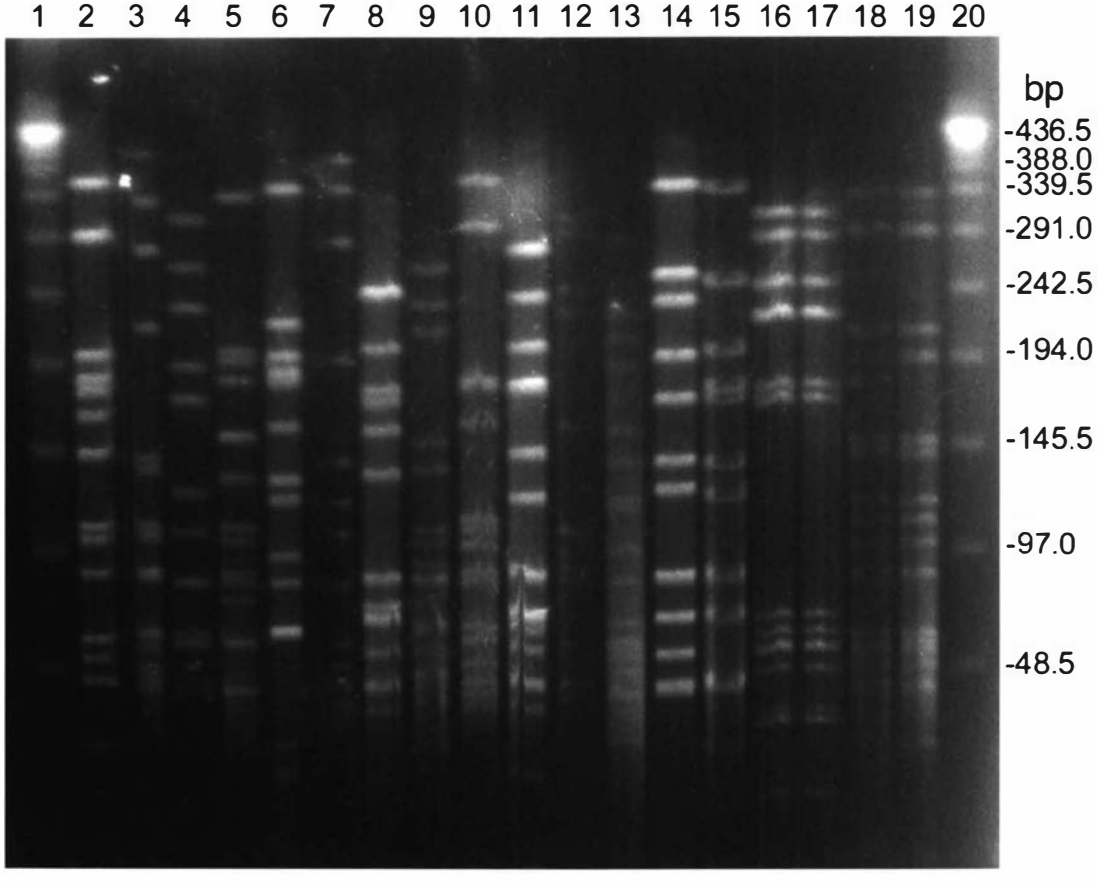
the isolates had no band location in common the degree of dissimilarity would be equal to one. Dendrograms were used to graphically express the genetic similarity of different subsets of isolates.

Results

The 343 *S. uberis* isolates (including the ATCC and reference strains) could be divided into 330 REA patterns. The ATCC isolate from the United States of America (USA) had a REA pattern unlike any of the isolates from New Zealand. Figure I is a graphic image of the Polaroid picture taken of one of the gels analysed. In lanes 1 and 20 is the marker. The band with the least base pairs (bp) migrated the furthest (most distal) through the gel (Figure I). The lightest band in the marker is a band with 48.5 bp. The band, which migrated least (most proximal), was the band with 533.5 bp. The similar migration of the marker on either end of the gel verified the uniformity of thickness of the gel on either end. If one end of a gel was thicker than the other end, the band locations would have been shifted proximally, due to a slower migration. The location of the marker bands can be used to estimate the size of the bands of the isolates tested on the same gel. In lane 2 is the reference strain #13. A variation of REA patterns is exhibited in the duplicate pairs of isolates taken from the same quarter during the same sample period in lanes 3 through 10. These 4 pairs of duplicate pairs' REA patterns differ from each other by at least 7 band locations. Lanes 11 through 13 contain isolates from different cows on different farms. An identical REA pattern is noted in lanes 16 and 17, which contain the same isolate #186. Lanes 18 and 19 contain two different isolates, which differ by one band location. The isolate in lane 18 contains a band between 194.0 and 145.5 bp, which is missing from the isolate in lane 19.

Figure I: Pulsed-field electrophoresis gel.

Figure I: Pulsed-field electrophoresis gel.



Index: Figure I: Pulsed-field electrophoresis gel.

Lane # (from right)	Index #	Farm	Cow ID	District*	Sample Date	Infection
1	Marker					
2	13 (427)	B	34	Manawatu	31.10.96	Subclinical
3	55	E	3[a]	Manawatu	23.05.96	Subclinical
4	56	E	3[a]	Manawatu	23.05.96	Subclinical
5	57	E	16[b]	Manawatu	23.05.96	Subclinical
6	58	E	16[b]	Manawatu	23.05.96	Subclinical
7	63	E	76[c]	Manawatu	23.05.96	Subclinical
8	64	E	76[c]	Manawatu	23.05.96	Subclinical
9	67	E	106[d]	Manawatu	23.05.96	Subclinical
10	68	E	106[d]	Manawatu	23.05.96	Subclinical
11	59	E	52	Manawatu	23.05.96	Subclinical
12	61	E	55	Manawatu	23.05.96	Subclinical
13	71	E	135	Manawatu	23.05.96	Subclinical
14	677	D	412	Manawatu	02.10.97	Subclinical
15	688	S	99	South Taranaki[f]	04.11.97	Subclinical
16	186	E	16	Manawatu	23.05.96	Subclinical
17	186	E	16	Manawatu	23.05.96	Subclinical
18	701	R	999	Manawatu	10.11.97	Clinical
19	702	R	652	Manawatu	24.11.97	Clinical
20	Marker					

a = duplicate samples
b = duplicate samples
c = duplicate samples
d = duplicate samples
e = duplicate samples
*** all in the Wellington region, except isolate in lane 15**
f = Taranaki region

The gels were analysed using the type strain (#427) for reference. The results for band differences in REA patterns are presented in Tables III and IV. If two or more of the isolates had the same REA pattern, then the band difference was zero (Table III). Only the isolates with less than 7 band differences are represented in the tables.

Dendrograms (Figures II through IX) of eight farms depicts the dissimilarity of the isolates collected from the individual farms. Only the isolates collected from 8 farms were reported.

Table III: Cluster of isolates with less than 4 band differences

Isolates	Clinical status	District	Band difference	Characteristic of samples
13	subclinical	Manawatu	0	different cows from different districts
40	subclinical	Horowhenua		
36	subclinical	Manawatu	0	duplicate samples taken on the same day for the same quarter
37	subclinical	Manawatu		
41	subclinical	Horowhenua	0	different cows from different districts
43	subclinical	Manawatu		
46	subclinical	Manawatu	0	different cows from same farm, same collection day
47	subclinical	Manawatu		
54	subclinical	Manawatu	0	different cows, different districts, different islands
320	clinical	Hurunui		
85	subclinical	Manawatu	0	different cows, same farm, different collection day
87	subclinical	Manawatu		
112	clinical	Southland		different cows, same district
113	clinical	Southland	0	
114	clinical	Southland		
277	subclinical	Manawatu	0	duplicate samples taken on the same day from same quarter
278	subclinical	Manawatu		
279	subclinical	Manawatu	0	duplicate samples taken on the same day from same quarter
280	subclinical	Manawatu		
281	subclinical	Manawatu	0	duplicate samples taken on the same day from same quarter
282	subclinical	Manawatu		
329	clinical	Carterton	0	different cows from different districts
330	clinical	So. Taranaki[a]		
334	clinical	Manawatu	0	same cow, different quarters collected on the same day
336	clinical	Manawatu		
57	subclinical	Manawatu	1	duplicate samples taken on the same day from same quarter
58	subclinical	Manawatu		
214	clinical	So. Taranaki[a]	1	different cows from different districts
215	clinical	Manawatu		
220	clinical	Manawatu	1	different cows from same farm, different collection day
234	clinical	Manawatu		
162	clinical	Manawatu	3	different districts
163	clinical	So. Taranaki[a]		

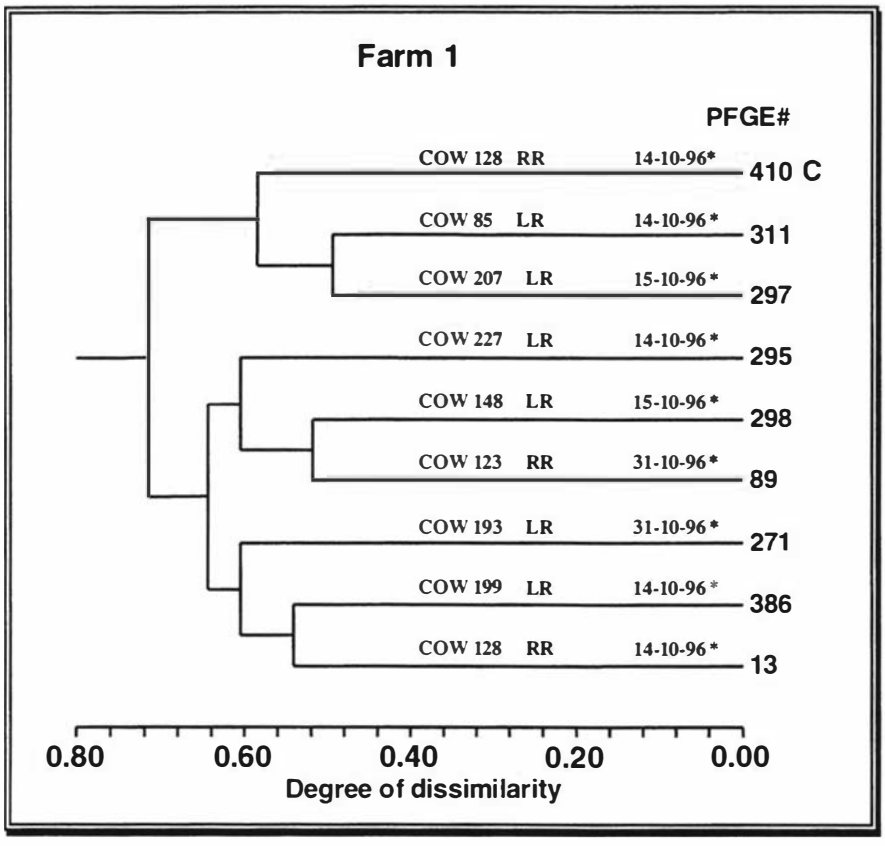
a = South Taranaki

Table IV: Cluster of isolates with 4 to 6 band differences

Isolates	Clinical status	District	Band difference	Characteristic of samples
100	clinical	Far North	4	different districts
102	clinical	Manawatu		
153	subclinical	Manawatu	4	different farms, same districts
344	clinical	Manawatu		
334	clinical	Manawatu	4	same cow, different quarters collected on the same day
335	clinical	Manawatu		
336	clinical	Manawatu		
104	clinical	Waikato	5	different farms, different districts, different regions
150	clinical	Manawatu		
350	clinical	Central H. Bay	5	different districts
356	clinical	So. Taranaki		
651	subclinical	Manawatu	5	different cows, same farm, collected on the same day
652	subclinical	Manawatu		
54	subclinical	Manawatu	6	different districts
320	clinical	Hurunui		
74	clinical	New Plymouth	6	different districts
246	clinical	Matamata		
90	subclinical	Manawatu	6	different farms, same district
154	subclinical	Manawatu		
112	clinical	Gore	6	two from same district, one from different district
114	clinical	Gore		
134	subclinical	Whangarei		
115	clinical	Clutha	6	different districts
375	clinical	Manawatu		
143	clinical	Matamata	6	different districts
355	clinical	Manawatu		
162	subclinical	Manawatu	6	different farms, two from same district
178	subclinical	So. Taranaki		
198	clinical	Manawatu		
288	subclinical	Manawatu	6	different cows, same farm, same collection day
289	subclinical	Manawatu		
347	clinical	Manawatu	6	different districts
391	clinical	So. Waikato		

Figures III through X are dendrograms depicting the degree of dissimilarity of REA patterns of the tested isolates obtained from milk samples taken on the same day (Figure X) to samples taken from the same farm over a period of approximately a year (Figure VIII). Tables V through XII are the antibiogram patterns (see Chapter 6) of the isolates from those farms reported.

Figure II: The degree of dissimilarity of the banding patterns of the isolates from Farm 1 in the Manawatu district, Wellington Region.



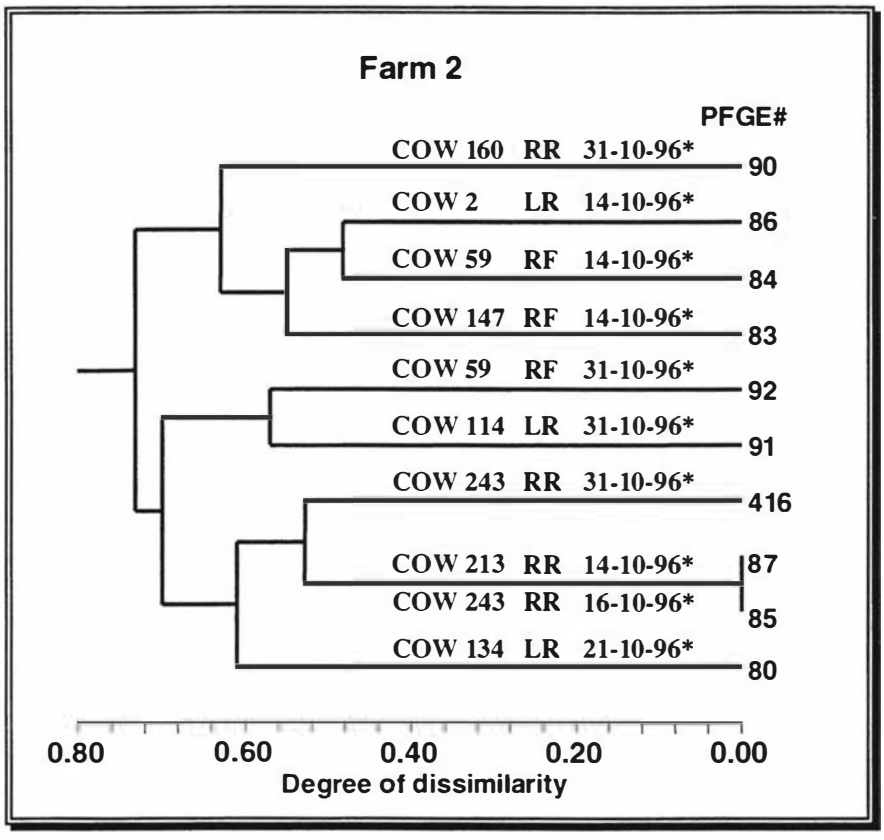
PFGE# = the pulsed-field gel electrophoresis number
* sample collection date
C=clinical isolates; all else subclinical isolates
RR = right rear quarter; LR = left rear quarter

Table V: Antibiogram patterns of the isolates from Farm 1 in the Manawatu district, Wellington Region.

PFGE#	Antibiogram Pattern[a]
13 -	C
89 -	C
271 -	D
295 -	C
297 -	C
298 -	C
311 -	C
386 -	C
410 -	C

a = antibiogram patterns for these isolates determined in Chapter 6

Figure III: The degree of dissimilarity of the banding patterns of the isolates from Farm 2 in the Manawatu district, Wellington Region.



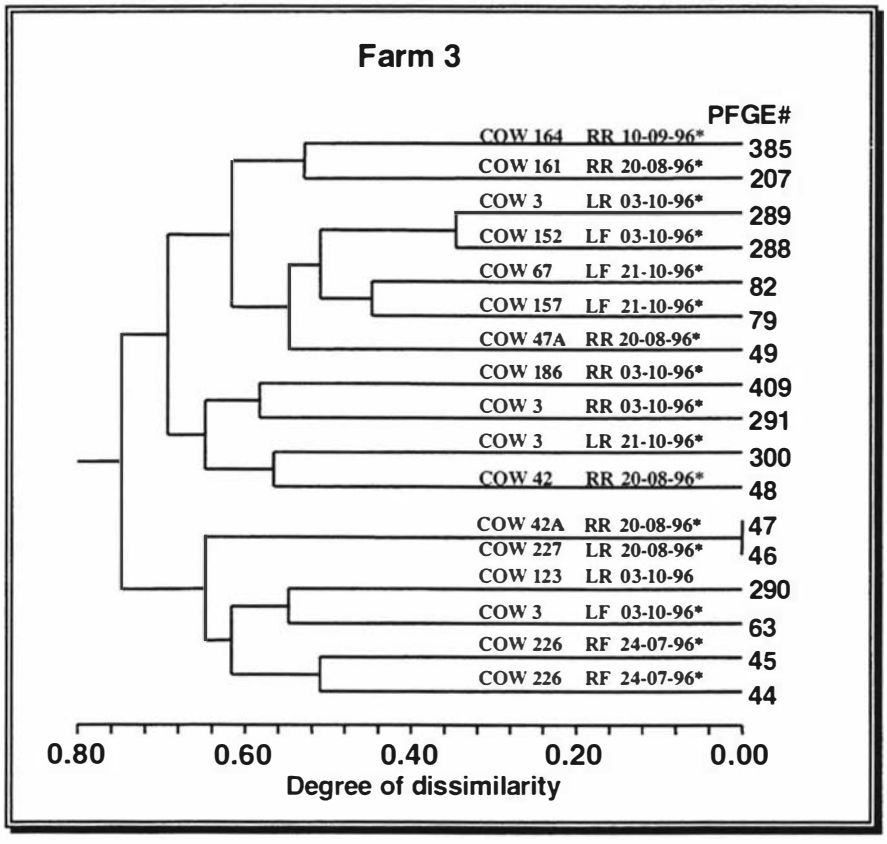
PFGE# = the pulsed-field gel electrophoresis number
* sample collection date
All subclinical isolates
RR = right rear quarter; LR = left rear quarter; RF = right front quarter

Table VI: Antibiogram patterns of the isolates from Farm 2 in the Manawatu district, Wellington Region.

PFGE#	Antibiogram Pattern[a]
80 -	C
83 -	C
84 -	C
85 -	D
86 -	C
87 -	C
90 -	C
91 -	C
92 -	I
416 -	C

a = antibiogram patterns for these isolates determined in Chapter 6

Figure IV: The degree of dissimilarity of the banding patterns of the isolates from Farm 3 in the Manawatu district, Wellington Region.



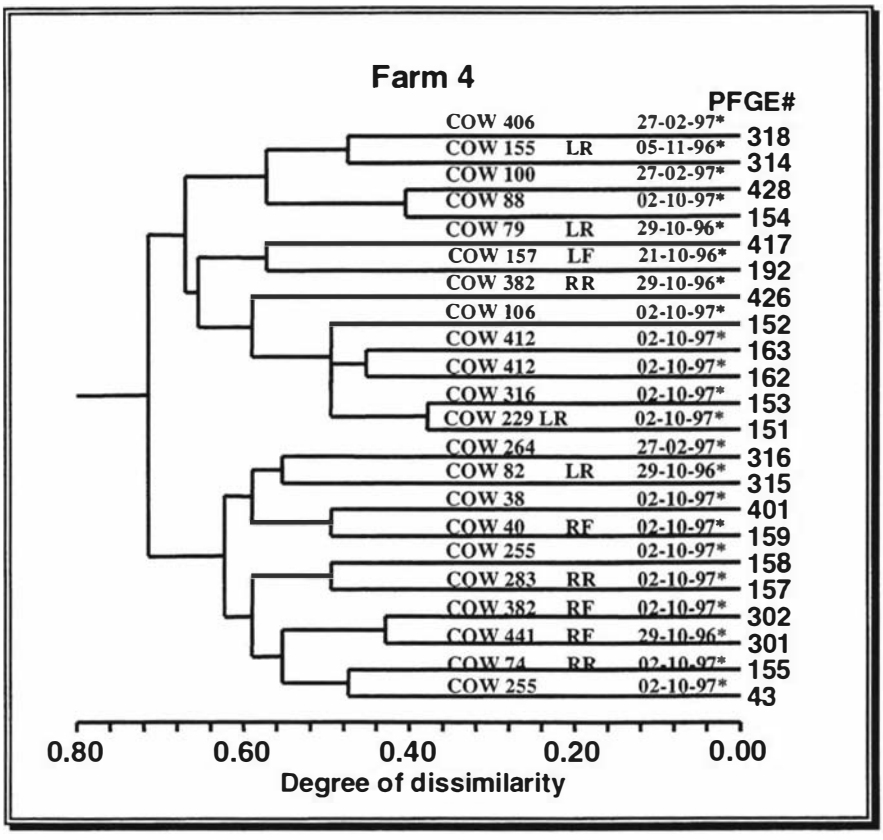
PFGE# = the pulsed-field gel electrophoresis number; cow numbers followed by an "A" are one of a duplicate sample taken at the same time period.
* sample collection date
All subclinical isolates
RR = right rear quarter; LR = left rear quarter; RF = right front quarter; LF = left front quarter

Table VII: Antibiogram patterns of the isolates from Farm 3 in the Manawatu district, Wellington Region.

PFGE#	Antibiogram Pattern[a]	PFGE#	Antibiogram Pattern[a]
44 -	C	207 -	C
45 -	C	288 -	*
46 -	C	289 -	C
47 -	*	290 -	I
48 -	F	291 -	C
49 -	C	300 -	C
63 -	C	385 -	A
79 -	C	409 -	A
82 -	*		

a = antibiogram patterns for these isolates determined in Chapter 6
* antibiogram pattern not determined

Figure V: The degree of dissimilarity of the banding patterns of the isolates from Farm 4 in the Manawatu district, Wellington Region.



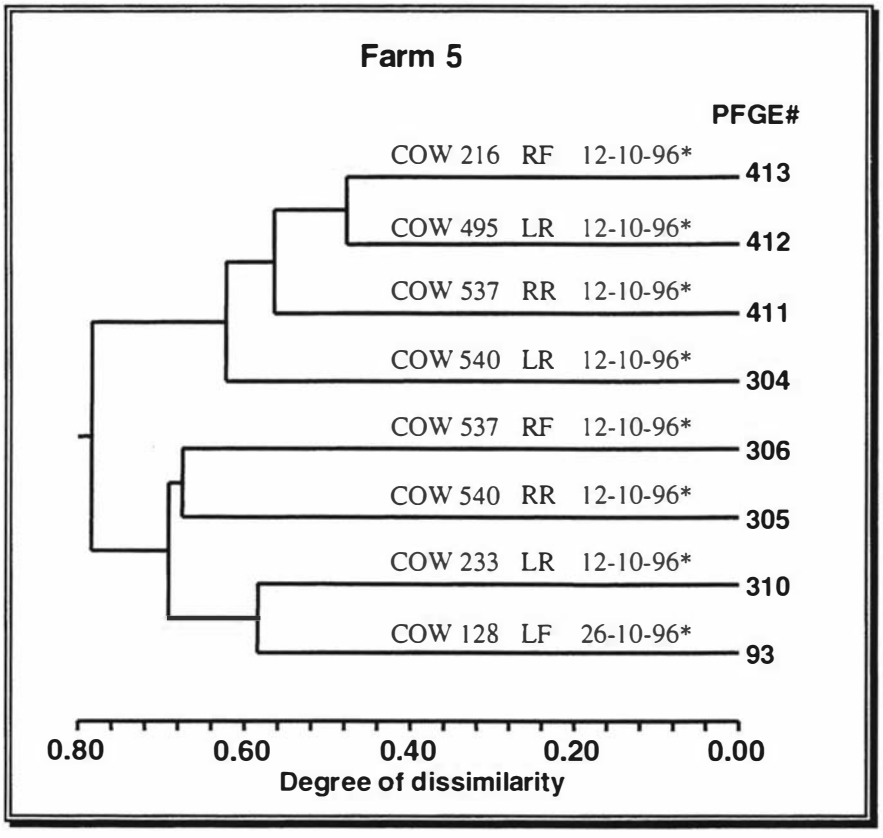
PFGE# = the pulsed-field gel electrophoresis number
* sample collection date
All subclinical isolates
RR = right rear quarter; LR = left rear quarter; RF = right front quarter; LF = left front quarter

Table VIII: Antibiogram patterns of the isolates from Farm 4 in the Manawatu district, Wellington Region.

PFGE#	Antibiogram Pattern[a]	PFGE#	Antibiogram Pattern[a]	PFGE#	Antibiogram Pattern[a]
43 -	C	159 -	C	315 -	C
151 -	C	162 -	*	316 -	C
152 -	C	163 -	C	318 -	C
153 -	C	192 -	C	401 -	C
154 -	C	301 -	C	417 -	C
155 -	P	302 -	C	426 -	C
157 -	C	314 -	I	428 -	C
158 -	C				

a = antibiogram patterns for these isolates determined in Chapter 6
* antibiogram pattern not determined

Figure VI: The degree of dissimilarity of the banding patterns of the isolates from Farm 5 in the South Taranaki Region, Taranaki Region.



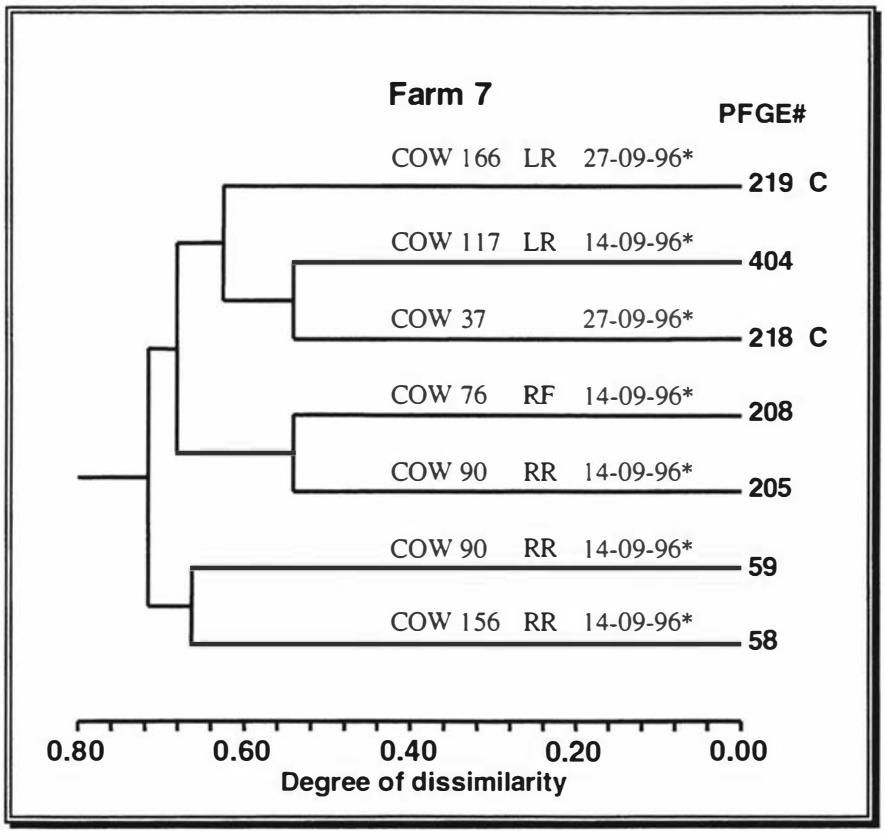
PFGE# = the pulsed-field gel electrophoresis number
* sample collection date
All subclinical isolates
RR = right rear quarter; LR = left rear quarter; RF = right front quarter; LF = left front quarter

Table IX: Antibiogram patterns of the isolates from Farm 5 in the South Taranaki district, Taranaki Region.

PFGE#	Antibiogram Pattern[a]
93 -	I
304 -	C
305 -	C
306 -	G
310 -	C
411 -	I
412 -	I
413 -	C

a = antibiogram patterns for these isolates determined in Chapter 6

Figure VII: The degree of dissimilarity of the banding patterns of the isolates from Farm 7 in the South Taranaki district, Taranaki Region.



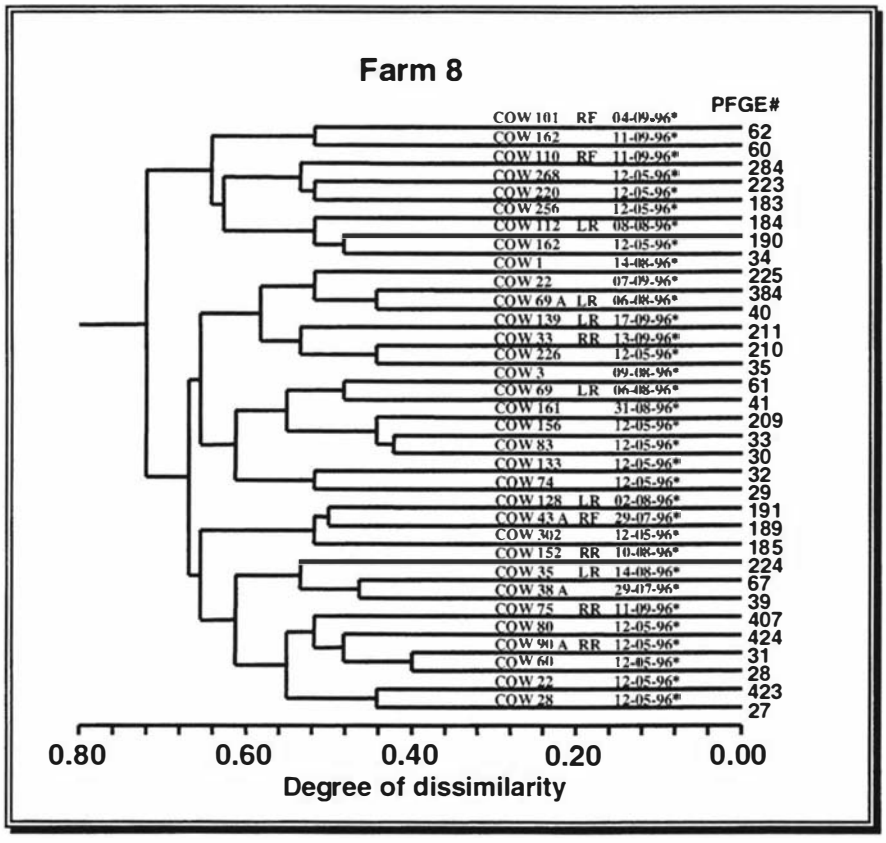
PFGE# = the pulsed-field gel electrophoresis number.
* sample collection date
C = clinical isolates; all else subclinical isolates
RR = right rear quarter; LR = left rear quarter; RF = right front quarter

Table X: Antibiogram patterns of the isolates from Farm 7 in the South Taranaki district, Taranaki Region.

PFGE#	Antibiogram Pattern[a]
58 -	*
59 -	C
205 -	C
208 -	C
218 -	C
219 -	H
404 -	D

a = antibiogram patterns for these isolates determined in Chapter 6
* antibiogram pattern not determined

Figure VIII: The degree of dissimilarity of the banding patterns of the isolates from Farm 8 in the Horowhenua district, Wellington Region.



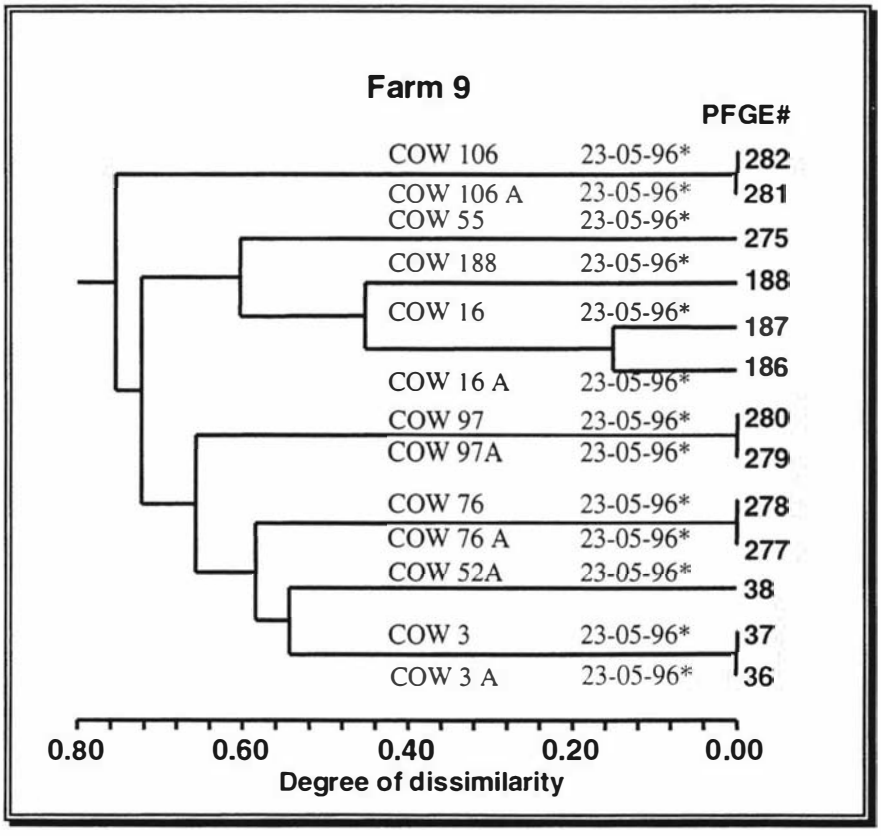
PFGE# = the pulsed-field gel electrophoresis number; numbers followed by an "A" are one of a duplicate sample taken at the same time period.
* sample collection date; all subclinical isolates
RR = right rear quarter; LR = left rear quarter; RF = right front quarter

Table XI: Antibiogram patterns of the isolates from Farm 8 in the Horowhenua district, Wellington Region.

PFGE#	Antibiogram Pattern[a]	PFGE#	Antibiogram Pattern[a]	PFGE#	Antibiogram Pattern[a]
27-	A	41-	C	209-	C
28-	C	60-	C	210-	C
29-	C	61-	*	221-	D
30-	D	62-	C	223-	*
31-	B	67-	C	224-	C
32-	C	183-	E	225-	C
33-	C	184-	D	284-	C
34-	*	185-	C	384-	L
35-	*	189-	C	407-	C
39-	C	190-	*	423-	A
40-	H	191-	D	424-	A

a = antibiogram patterns for these isolates determined in Chapter 6
* antibiogram pattern not determined

Figure IX: The degree of dissimilarity of the banding patterns of the isolates from Farm 9 in the Manawatu district, Wellington Region.



PFGE# = the pulsed-field gel electrophoresis number; numbers followed by an "A" are one of a duplicate sample taken at the same time period.
* sample collection date
All subclinical isolates

Table XII: Antibiogram patterns of the isolates from Farm 9 in the Manawatu district, Wellington Region.

PFGE#	Antibiogram Pattern[a]	PFGE#	Antibiogram Pattern[a]
36 -	*	275 -	C
37 -	C	277 -	C
38 -	A	278 -	C
186 -	*	279 -	A
187 -	*	280 -	C
188 -	*	281 -	C
		282 -	D

a = antibiogram patterns for these isolates determined in Chapter 6
* antibiogram pattern not determined

Discussion

Tenover *et al.* (1995) interpreted the chromosomal DNA patterns of their tested isolates based on the divergence of the patterns from that of their selected type strain. Using the criteria set forth by Tenover *et al.* (1995) and Fenwick (1997), the results of this study identify 11 pairs and one group of three of genetically identical strains (Table III). Four pairs of the consecutive duplicate milk samples taken on the same day from the same quarter had identical REA patterns (Table III). One of the consecutive duplicate pairs differed by one band and the other three pairs differed by at least 7 bands. This latter finding suggests that at least two genetically different *S. uberis* strains were isolated from a single quarter during the same sampling period. When the colonies were selected off the original culture plate for isolation, only one representative colony, which was grossly identical to the others, was selected for further identification. If all the colonies from the initial culture were tested, the number of genetically different strains identified may have been greater. In many cases, 50 or more grossly identical samples were grown on the initial culture; therefore, testing of every individual colony was not done, due to time, logistic and financial constraints.

The present results identified identical patterns within the same quarter of an individual cow, different quarters from the same cow, different cows within the same farm, and from different cows from the same or different districts, farming regions (Appendix III, figure 2, lanes 3, 4, and 5) or islands (Table I). The identical REA patterns were represented by isolates that were both from subclinical and clinical cases

(or three clinical cases – isolates #112, #113, #114). The exceptions were isolates #54 and #320. Isolates #54 and #320 were from different districts and #54 was from a subclinical case of mastitis and #320 from a clinical case. These were the only genetically identical isolates from different islands.

Baseggio *et al.* (1997) also described the clonal relationship of streptococcal isolates from mastitis cases, based on their REA patterns. They visually examined the restriction patterns of 21 *S. uberis* isolates from 10 herds and suggested a clonal relationship existed between 2 isolates from one herd because the pattern appeared identical and 2 isolates from another herd because their pattern only differed by one extra band. Three pairs of the present isolates examined had patterns, which differed by only one band. One was a duplicate pair from the same quarter and one pair was from different cows on the same farm. The third pair exhibited a clonal relationship between two isolates from different farming regions on the same island. Tenover *et al.* (1995) and Fenwick (1997) agree that if the REA patterns only differ by one to three bands from the type strain, then the isolates would be closely related by lineage, but genetically different. Fenwick (1997) classified these latter isolates as pulsotypes. They (Tenover *et al.*, 1995; Fenwick, 1997) further hypothesised that a two to three band difference in a REA pattern correlates to a change in the genome due to a genetic event (i.e. a deletion, insertion, or a point mutation).

The isolates of this study having REA patterns, which differed by four to six bands (Table IV), were possibly related as defined by Tenover *et al.* (1995) and Fenwick (1997). The remainder of the isolates would be considered different strains because their REA patterns differ by more than seven band locations (Tenover *et al.*, 1995 and

Fenwick, 1997).

Tenover *et al.* (1995) suggested that the interpretation of DNA restriction patterns produced by PFGE could be used in the examination of the epidemiology of a short-term outbreak of a disease. The genetic guidelines (Figure X) for determining relatedness was to be used for sample sizes of ≤ 30, collected during a 1-3 month period (Tenover *et al.*, 1995).

Figure X: The criteria used by Tenover *et al.*, 1995 for interpretation of DNA restriction patterns with regards to disease outbreaks

Indistinguishable = genetically identical (part of the outbreak) Closely related = occurrence of one genetic event, 2-3 bands difference, probably part of the outbreak Possibly related = occurrence of two genetic events, 4-6 bands difference, possibly part of the outbreak Different = occurrence of three or more genetic events, ≥7 bands difference, not part of the outbreak

Isolates from farms 1, 2, 3, 5, and 9 (Figures II, III, IV, VI, IX, respectively) were obtained over a period of less than 3 months, but only farm 3 would fulfil the criteria set forth by Tenover *et al.* (1995). On Farm 3 (Figure V) only one pair of isolates (#46 and #47) had a degree of dissimilarity equal to zero. The REA patterns of these isolates are displayed in Appendix III, figure 3, lanes 6 and 7. These genetically identical isolates came from two different cows collected on the same day. These two cows were infected by the same genetic strain from the same source or through cow to cow contact. One was isolated from the left and the other the right side of the udder, which would negate the possibility of teat cup contamination, contrary to the two infected right rear quarters on Farm 2 (#85 and #87, Figure III). The remainder of the isolates on this farm differed by at least 30%. The isolates #288 and #289 (Figure IV) REA patterns differed by 6 bands, which coincides to approximately a 33% or .33

degree of dissimilarity. This would indicate isolates #288 and #289, which were from two different cows, are possibly related and from the same source, but the other isolates obtained were not related to any other isolates from this farm. Isolates #44 and #45 (Figure IV) were from duplicate milk samples taken during the same sampling period. Those two isolates had a degree of dissimilarity of approximately 50%. That particular quarter was infected by two different or unrelated strains of *S. uberis* from possibly the same or different sources. Cow 3 (Figure IV) had two unrelated strains (#289 and #291) isolated from two different quarters during the same sample period. A different strain (#300) from Cow 3 was isolated 18 days later. Isolate #300 was from the same quarter as isolate #289, but their degree of dissimilarity was at least 70%. The degree of dissimilarity between isolates #291 and #300 was less than that between the two isolates from the same quarter (#289 and #291). Isolate #289 may have undergone at least three genetic events, which resulted in the development of the new strain #300 or there may be no genetic lineage linking the two strains. Although there was a high degree of dissimilarity among the isolates from Farm 3, there were no two isolates that were 100% dissimilar.

The isolates from Farms 1, 2, and 5 (Figures II, III, VI respectively) were dissimilar by at least 45%, except for #85 and #87 (same REA pattern) from Farm 2. Farm 5 (Figure VI) had two pairs of isolates from different quarters on the same cow (#304 and #305; #306 and #411) which were 80% dissimilar. These pairs were isolated at the same sample period. Different or the same sources with multiple strains may have infected these quarters.

All the isolates collected on Farm 9 (Figure IX) were collected during the same sample period. Duplicate samples constituted the majority of the isolates cultured on Farm 9.

Four of the five duplicate pairs of isolates represented have a degree of dissimilarity equal to zero. The other duplicate pair has a band difference of 1 and a degree of dissimilarity equal to approximately 14%. On a single collection date eight genetically different *S. uberis* strains were isolated from 13 samples taken. Irrespective of the duplicate isolates, the isolates from this herd were dissimilar by at least 45%.

Streptococcus uberis isolates collected from different quarters on the same or different cows in a herd, during a period of less than 3 months may exhibit a degree of dissimilarity of at least 45%. These isolates would not be related and are not considered to be a part of an outbreak of mastitis on a farm, due to a common source.

The degree of dissimilarity in isolates collected over a longer period (5 months) of time exhibited at least 50% dissimilarity (Figure VIII). These results would be expected, since there was a high degree of dissimilarity in an individual herd where samples were collected within a shorter period of time. A longer time between samples would allow more time for genetic events to occur, allowing for a greater degree of dissimilarity.

Two hundred and eighty-three of the 342 isolates were not possibly related. This represents a vast heterogeneity of the *S. uberis* strains isolated from 35 different districts, from 18 different farming regions, in New Zealand. These results confirm the diversity noted in 21 *S. uberis* isolates tested by Baseggio *et al.* (1997). However, Baseggio *et al.* (1997) only sampled from two herds in their study. The lack of any real pattern of similarity between substantial numbers of isolates indicates that infection with *S. uberis* does not appear to be due to some characteristic of a particular strain of the organism causing an outbreak of contagious infection. The finding

supports the view that infection with *S. uberis* in dairy cows in New Zealand is largely due to opportunistic infection with a great variety of strains, which are present on cows and in their immediate environments. The finding of a few cases of identical or very similar strains within cows in different quarters or in different cows on the same farms suggest that cows may be infected by the same organism either from a common source or by spread from one quarter or cow to another.

The multitude of strains of *S. uberis* isolates strengthens the classification of *S. uberis* as an environmental pathogen with multiple environmental habitats. Prevention of mastitis caused by *S. uberis* would include the minimisation of exposure to the pathogens, hygienic milking procedures and a possible vaccination protocol. The great genetic diversity of strains in this case does not necessarily correlate to the effectiveness of a vaccine directed at surface antigens. The surface antigens of these strains may be homogeneous, regardless of the heterogeneous REA patterns. The identification of a common antigen on the surface of the *S. uberis* isolates would be useful for the development of a vaccine directed towards that antigenic determinant. An example of a common antigen produced by a majority of *S. uberis* isolates from clinical cases of bovine mastitis would be PauA (Leigh, 1997). PauA activates plasminogen to plasmin. A vaccine directed towards PauA may retard the growth of many different strains of *S. uberis* isolates allowing time for the natural immune system to eliminate the infection. Leigh (1997) noted quarters from animals immunised with 100 μg/ml of PauA antigen shed less bacteria after an experimental challenge of *S. uberis* than those immunised with 0.1 μg/ml PauA antigen.

A New Zealand dairy farmer can adopt minimisation of exposure and hygienic milking

procedures, but no vaccine directed against *S. uberis* mastitis is presently available. If the surface antigens are as heterogeneous as the DNA, with no common surface antigen, then an effective vaccine will have to be one, which will be effective against a multitude of strains. Even an autogenous vaccine developed for a particular farm would need to be cross protective against a variety of strains. An autogenous vaccine for Farm 9 would need to be effective against at least 8 possible different stains of *S. uberis* on that farm and Farm 8 would require a vaccine effective against at least 33 strains. The more *S. uberis* isolates that were obtained the more different strains were noted on the individual farms. The degree of dissimilarity on farms where 20 or more isolates were obtained (Farm 4, n = 20 and Farm 8, n = 33) was at least 35%. The dissimilarity of at least 35% implies all the isolates are different, not clonally related.

An effective autogenous vaccine for Farm 1 (Figure II) would need to contain at least 9 different strains, but an antibiotic regimen that incorporated the use of the antibiotics that the strains were sensitive to, has the potential to eliminate 8 of the 9 strains reported. The 8 strains (Table V) are of the pattern C antibiogram (see Chapter 6). Pattern D (see Chapter 6) differs from pattern C by an additional antibiotic resistance to tetracycline. Similar results are reported in the other farms. No individual farm had more antibiogram patterns than PFGE patterns. Although a standard antibiotic treatment regimen may appear more effective at elimination of multiple strains of *Streptococcus uberis* on a particular farm, treatment of subclinical cases during the lactation period is not economical (see Chapter 5).

If an outbreak of *S. uberis* mastitis were to occur, PFGE and antibiograms, in combination, would be useful techniques for epidemiological studies (Sloos *et al.*, 1998). Pulsed-field gel electrophoresis alone is a highly discriminatory test

(Bannerman *et al.*, 1995; Fenwick 1997; Sloos *et al.*, 1998), but the technique requires 5-6 days to obtain a result. Antibiograms are easier and quicker to perform, but alone are not very discriminatory. The greater number of REA patterns on an individual farm compared to the number of antibiogram patterns would indicate a greater discriminatory capability of the PFGE to subdivide the *S. uberis* isolates within a farm.

There is minimum variability in the antibiogram patterns. Most of the isolates are of the antibiogram pattern C. Two isolates that were genetically identical according to the REA patterns (#85 and #87, Figure III) had different antibiogram patterns. It is easier to obtain a false positive with the antibiogram typing technique (see Chapter 6) than with the PFGE. The PFGE was more sensitive than the antibiogram typing technique used in Chapter 6. A standard marker is used with every PFGE run. A standard isolate was not put on every agar plate to test antibiotic sensitivities. If the antibiotic disc used did not contain a sufficient amount of antibiotic, it could result in a false negative (not truly resistant to the antibiotic tested). If isolate #271 (Figure II) was sensitive to tetracycline then it would have had the pattern C antibiogram. All the isolates from Farm 1 (Figure II) could not be discriminated on the basis of the antibiogram patterns; although the PFGE indicates they are not related.

The results presented confirm the presence of a multitude of genetically different strains of *S. uberis* from different islands, farming regions, districts, farms and from within a cow, which may be a result of relatively small changes at the chromosomal level. A high degree of dissimilarity of strains was noted on farms where samples were collected during a period from 3 months up to a 2 year (this farm not individually reported) collection period. Like with other environmental pathogens, prevention will be the key to minimise the incidence of mastitis cause by *S. uberis*.

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Conclusion

"Even cows on greener pastures get mastitis"

Conclusion

Feeding and management of dairy cows on pasture is the New Zealand way of milk production. Under seasonal pasture grazing management practices, dairy cows may experience cases of subclinical or clinical mastitis caused by a multitude of potential pathogens, in particular, *Streptococcus* and *Staphylococcus* spp. (Chapter 5). Mastitis causes milk production losses, costs of treatment, and other monetary losses related to the prevalence of mastitis. Mastitis should be prevented before these economic losses are incurred.

A mastitis prevention programme, Seasonal Approach to Managing Mastitis (SAMM) Plan, was designed during 1990/91. The SAMM Plan is a dynamic programme designed to improve the udder health in New Zealand dairy herds, producing milk seasonally from grazed pastures. The programme suggests management practices to minimise and/or prevent the incidence of mastitis in herds. The programme is under constant revision with the development of new techniques to diagnose, treat, and prevent mastitis. Useful information from trials performed under New Zealand conditions are incorporated into the Plan.

Table I: The main points to the 1994/95 SAMM Plan (Anon, 1995)

Late lactation period	⇒	***Review clinical cases*** ***Review cow counts*** ***Treat late season clinicals*** ***Decide dry cow antibiotic strategy***
Drying-off period	⇒	***Dry-off abruptly*** ***Administer dry cow antibiotics*** ***Check quarters***
Dry period	⇒	***Test machine and correct faults*** ***Treat clinicals*** ***Enrol for somatic cell counting*** ***Set up recording system***
Calving period	⇒	***Use clean pasture*** ***Treat and record clinicals*** ***Teat spray*** ***Milk out completely*** ***Minimise suckling***
Lactation period	⇒	***Monitor machine and correct faults*** ***Treat clinicals*** ***Monitor bulk somatic count*** ***Practise good hygiene*** ***Use good milking technique*** ***Teat spray*** ***Manage teat condition***

Some recommendations in the Plan were initially promoted without significant supporting research. The present thesis was undertaken to strengthen the scientific basis for the SAMM Plan. It aimed to extend scientific research into some recommendations in the Plan that previously had an inadequate scientific basis and to test the recommendations within controlled scientific studies. The first study (drying-off procedures) was initiated because several farmers wanted to know what is the ideal procedure to dry-off cows, to minimise the prevalence of mastitis.

Drying-off period

Table II: Drying-off period control activities (modified chart, Anon, 1995)

Correct drying-off technique	• ***Reduce feeding*** • ***Continue to spray teats*** • ***Abrupt cessation of milking*** • ***No intermittent milking***
Dry cow therapy	• ***After last milking of lactation*** • ***Use only at drying-off*** • ***Sanitise teat ends with meths before treatment*** • ***Leave some antibiotic in teat canal*** • ***Spray teats***
Examine cows regularly	• ***Bring in herd*** • ***Periodically palpate all quarters*** • ***Start 7 days after drying-off*** • ***Weekly intervals for minimum of 3 weeks*** • ***Do not break teat seal*** • ***Don't remove milk or secretion unless clinical*** • ***Clinicals – strip quarter out completely and administer lactation antibiotic using full course of treatment (veterinarian advice recommended)*** • ***Record clinical details***

The 1994/95 SAMM Plan (Anon, 1995) recommended abrupt cessation of milking on the last day of the lactation period, without intermittent milking (drying-off period control activities). This recommendation was based on information extrapolated from a small number of trials performed in other countries, where generally, management of cows is quite different to that applied in New Zealand. Despite this recommendation, some farmers continued to perform intermittent drying-off procedures to conform to their previous management. The present results (Chapter 4) indicate that drying-off procedures can affect the prevalence of mastitis at dry-off, but does not have a significant affect on the prevalence of mastitis in the first 12 days of the following

lactation. In this trial a significant number of mastitis quarters were culled due to other reasons not directly related to mastitis; therefore, this may have influenced the ability of the study to observe a true difference at early lactation. However, milking cows once every other day for the last 8 days of the lactation period increased the prevalence of quarter mastitis at dry-off compared to milking once daily over the same period.

Cows and heifers entering the early lactation period with mastitis was the next area to be studied.

Lactation Period

Table III: Lactation period control activities (modified chart, Anon, 1995)

Identify and Treat Clinicals	• *Udder swelling* • *Watch for clots on milk filter* • *Investigate abnormalities* • *Inspect foremilk to identify potential clinicals* • *Get milk samples taken for advice on correct treatment* • *Sanitise teat ends with meths before treatment* • *Use full course of antibiotics* • *Dry-off uncured quarters* • *Clearly mark treated quarters* • *Observe withholding time (penalties are severe)* • *Record all details*
Graph Bulk Tank Cell Counts	• *Identify suspect cows from cell counts* • *Consider strategic culling or dry off cows or quarters* • *Seek veterinarian advice and treat highest count cows* • *Check all control measures*

Identify and treat clinicals

Clinical mastitis occurs even in well managed herds operating effective mastitis control programs. To deal with this, the SAMM Plan recommended the identification and treatment of clinical infections (Table III). The treatment of clinical cases was not reviewed in detail in the present studies, but the antibiograms (Chapter 6) and REA (Chapter 7) patterns of *Streptococcus uberis* isolates from clinical cases were examined, because *Streptococcus uberis* has recently been recognised as an important cause of mastitis in New Zealand. In general, the clinical isolates were genetically different from one another as well as from the subclinical *Streptococcus uberis* isolates, but there was much less variability in the antibiogram patterns with close similarity between clinical and subclinical isolates. Seventy-five percent of the subclinical and 85% of the clinical isolates were of the antibiogram pattern C.

Graph bulk tank cell counts

The recommendation was to identify and treat suspected subclinical mastitis cases based on their individual cow somatic cell counts (Table III). The identification of infected quarters and the efficacy and cost effectiveness of treatment of cows with elevated cell counts during lactation were examined in Chapter 5. Bacteriological cultures, individual cow somatic cell counts, the electrical conductivity of the milk and the Rapid Mastitis Test (RMT) can be used to assist in the diagnosis of subclinical mastitis (Chapter 5). These "cow-side" tests are used in conjunction with known "elevated" individual cow somatic cell counts to detect affected quarters. It is recommended in the SAMM Monthly Mastitis Focus (Steffert, 1997) to use the RMT

and conductivity meters to detect affected quarters. Sixty-six percent of quarters infected by major pathogens were diagnosed correctly using electrical conductance and/or the RMT (Chapter 5).

After identifications of the subclinical cases are noted, their management will be dependent upon many factors including individual cow factors, the pathogen(s) involved and environmental factors. The costs and benefits from treating infected quarters during lactation (assuming current NZ costs and prices), plus the inevitable cost of treatment of uninfected quarters (which were falsely identified as type 1 errors or which spontaneously cured before the initiation of treatment), resulted in a net loss of approximately $9.18 per quarter treated (Chapter 5). However, under different conditions with different costs and prices, or where different antibiotics are used, there may be a benefit from treating subclinical cases. For example, if the effective antibiotic chosen for the treatment of the subclinical cases, described in Chapter 5, cost less than NZ 94 cents/unit of therapy, then there would be a net gain from treating subclinical mastitis during the lactation period (Chapter 5).

Streptococcus uberis

Bacteriological cultures of clinical cases are recommended for clinical infections (Table III) and may be used as a diagnostic technique for detection of subclinical mastitis (Chapter 5). *Streptococcus uberis* is a prominent major pathogen isolated from subclinical and clinical cases of mastitis in New Zealand dairy cattle. There was a high degree of variation (as determined by PFGE) among the *Streptococcus uberis* isolates studied in this work which were causing infections in New Zealand dairy cattle

(Chapter 7), which is an indication of the opportunistic nature of the organism in the dairy cows' environment. However, 80% of the isolates tested were of the antibiogram pattern C, which suggests a homogeneity, with respect to the *in vitro* sensitivity of the *Streptococcus uberis* isolates to the 12 antibiotics tested (Chapter 6). The antibiogram typing technique is of low discriminatory value (Aarestrup *et al.*, 1995), but it can be performed quickly and easily (Hsieh and Liu, 1995). Pulsed-field gel electrophoresis (PFGE) typing is a more discriminatory technique (Aarestrup *et al.*, 1995), but this technique requires 5-6 days to obtain a result and the equipment is expensive and it is not economically practical for it to be available in every commercial diagnostic laboratory and/or veterinary clinic. If a substantial number of milk samples are collected for diagnostic purposes, a laboratory can be set up in a veterinary practice to perform antibiotic sensitivity testing. Most bacteriological culture results can be obtained within 24 to 48 hours, when performed in the laboratory within the practice.

The diverse *Streptococcus uberis* genotypes isolated in New Zealand (Chapter 7) indicates the need for a diverse prevention and treatment programme. However, the lack of diversity in the antibiogram patterns (Chapter 6) of the isolates indicates that a standard antibiotic treatment protocol would be sufficient. However, mastitis caused by *Streptococcus uberis* continues to occur in farms using antibiotics noted to be effective at preventing the growth of *Streptococcus uberis in vitro* (Chapter 6), reinforcing that reliance on antibiotics alone may be inadequate in the control of mastitis. The *in vitro* response of a pathogen to an antimicrobial does not necessary equate to its *in vivo* response to that antimicrobial. Milk constituents, concurrent antimicrobials use, administration technique of the antimicrobial, environmental conditions and other cow and environmental factors can effect the *in vivo* response.

"So where do we go from here?"

Additional epidemiological information about *Streptococcus uberis* mastitis in New Zealand dairy cattle is required to better understand how to, first control then, if necessary, treat infections caused by the pathogen. An economical and easy typing technique is needed for rapid identification of the pathogen involved in mastitis infections. Since treatment of subclinical mastitis cases identified by techniques monitoring elevated cell counts during the lactation period is not economically viable (Chapter 5), the information gained from a very discriminatory typing technique could be used for epidemiological investigations. Determination of the source of the infection will provide a better understanding of the application of improved prevention measures.

Calving Period

Table IV: Calving period control activities (modified chart, Anon, 1995)

Calve cows in clean environment	• *Clean pasture* • *Clean raceways* • *Reduce exposure to environmental mastitis*
Watch for clinicals	• *Strip and examine foremilk daily while in colostrum mob* • *If milk grading problems investigate suspect cows* • *Where practical use separate mob and milk clinicals last* • *Check withholding time for antibiotics used*
Teat spray	• *Start at first milking and continue for all lactation* • *Used licensed teat spray with 15% emollient* • *Use 20% emollient if teat sore/damage problem*
Fast efficient milking	• *Ensure all quarters of all cows milked out* • *Ensure milk letdown particularly in heifers* • *Avoid both under milking and over milking*
Minimise suckling	• *Maximum of 24 hours suckling on dam* • *Remove calf and machine milk cow twice daily*
Graph Bulk Tank Cell Counts	• *Identify suspect cows from cell counts* • *Consider strategic culling or dry off cows or quarters* • *Seek veterinarian advice and treat highest count cows* • *Check all control measures*

Clean environment

What constitutes a "clean" environment (Table IV)? For example, how much pasture cover (kg DM/hectare) is needed to minimise the prevalence of mastitis at calving, to be considered a "clean" and a nutritionally "safe" environment?

Further investigation into mastitis prevention strategies during the calving period is needed, including the effect of isolation of "carrier" animals prior to calving, the role of application of pre-disinfecting solution and/or barrier dips prior to calving, minimising suckling of the calves, the application of pre-dipping with antiseptics as a preparation prior to milking, dipping versus spraying and fast efficient milking. These aspects were not examined in this thesis due to time constraints.

Antibiotic therapy

Streptococcus uberis isolates from New Zealand dairy cattle mastitis cases appear to be susceptible *in vitro* to most commercially available intramammary antibiotics labelled for the treatment of *Streptococcus uberis* and/or gram-positive bacteria (Chapter 6). Although genotypically very diverse (Chapter 7), the antibiotic resistance patterns among *Streptococcus uberis* isolates from both subclinical and clinical cases of mastitis are very similar. The isolates from clinical cases of mastitis are, however, more sensitive to cloxacillin than the subclinical isolates (Chapter 6). The antibiotic resistance patterns of *Streptococcus uberis* from New Zealand mastitis cases were different from those reported from the United States. Further investigation in the type

and duration of the most effective antibiotic for the elimination of *Streptococcus uberis* mastitis under New Zealand conditions is required.

Vaccines

The development of a vaccine against *Streptococcus uberis* is needed, although the diverse nature of *Streptococcus uberis* isolates in New Zealand and even within a farm would minimise the usefulness of a *Streptococcus uberis* vaccine developed from whole cell cultures. Further development of a vaccine directed at an individual *Streptococcus uberis* antigen is being studied (personal communication, Pankey JW, 1997), which may be effective in preventing and/or minimising *Streptococcus uberis* mastitis in New Zealand dairy cattle.

The continual influx of new research from the Dairying Research Corporation in Hamilton and Massey University, in the area of dairy cattle mastitis under New Zealand conditions will strengthen the benefit of the SAMM Plan for the improvement of udder health and milk quality in New Zealand.

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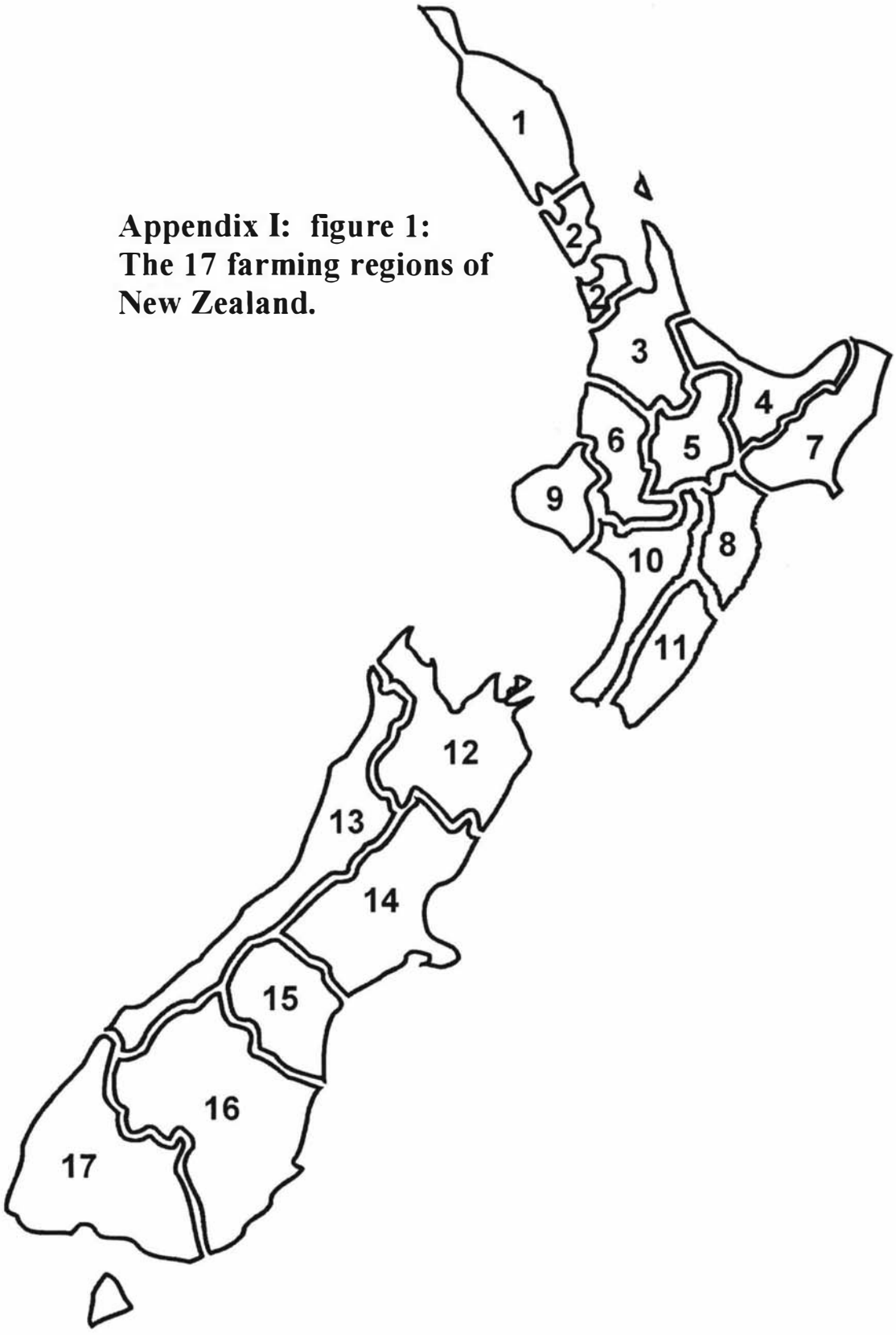
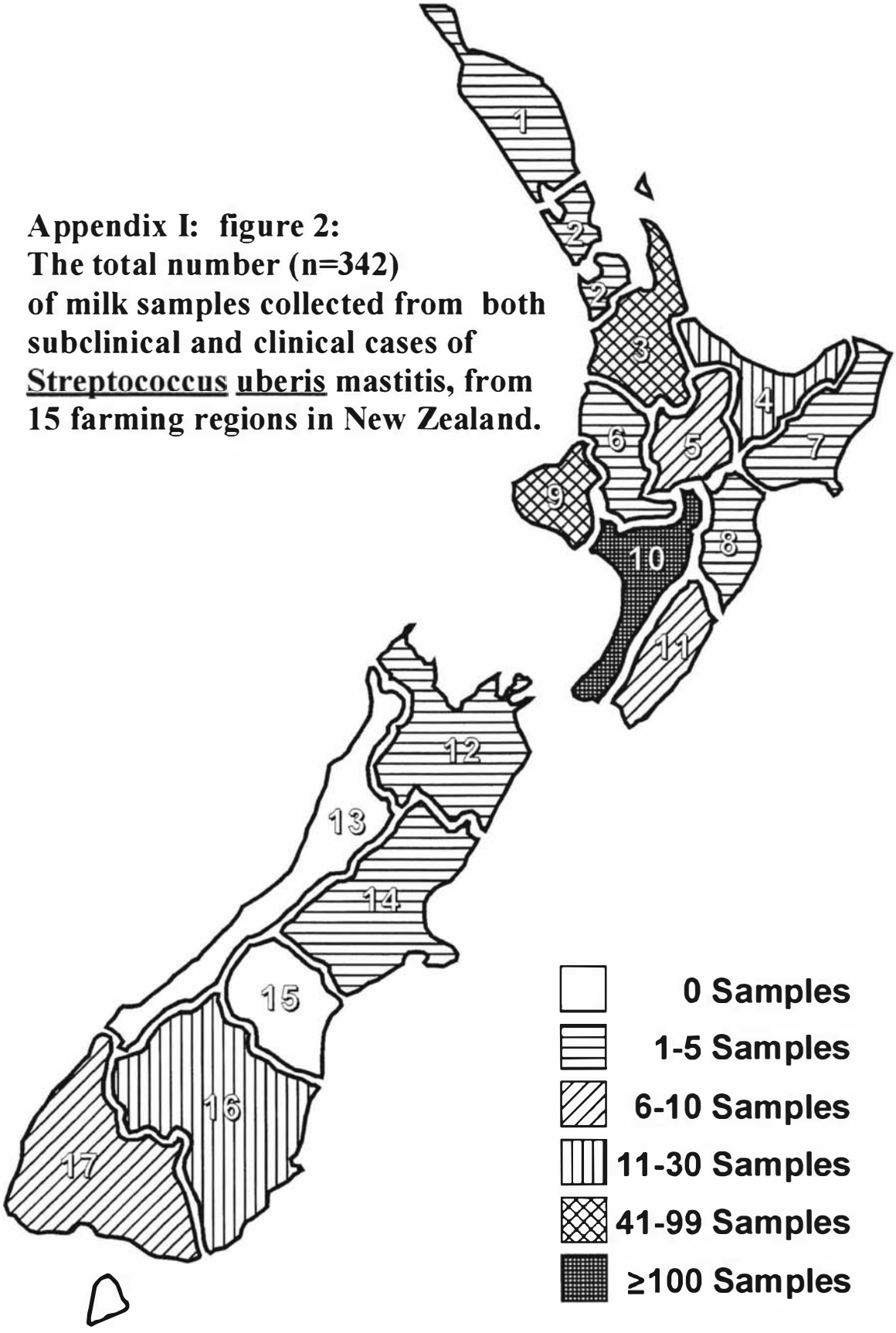
Legend: Appendix I: figures 1 - 4:

(1) Northland Far North Whangarei	***(10) Wellington*** Rangitikei Manawatu Horowhenua Upper Hutt
(2) Central Auckland Franklin	
(3) South Auckland Hauraki **Waikato** Matamata-Piako Waipa South Waikato	***(11) Wairarapa*** Tararua Carterton South Wairarapa
(4) Bay of Plenty Western Bay of Plenty Tauranga Whakatane	***(12) Nelson/Marlborough*** Kaikoura
(5) Central Plateau Rotorua	***(13) Westland***
(6) Western Uplands Waitomo	***(14) North Canterbury*** Hurunui
(7) East Coast Gisborne	***(15) South Canterbury***
(8) Hawkes Bay Central Hawkes Bay	***(16) Otago*** Central Otago Dunedin Clutha
(9) Taranaki New Plymouth South Taranaki	***(17) Southland*** Southland Gore

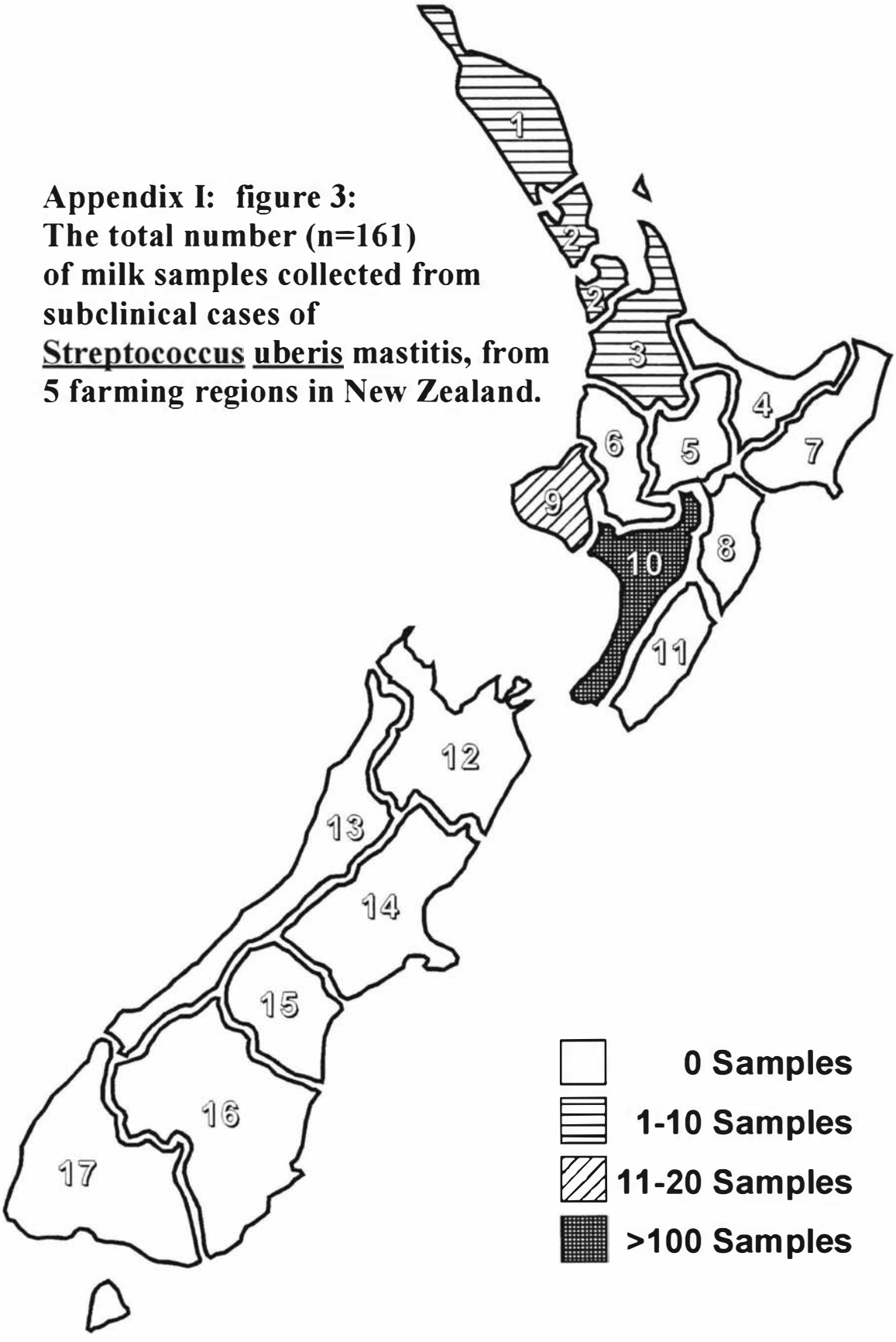
Appendix I: figure 1:
The 17 farming regions of New Zealand.



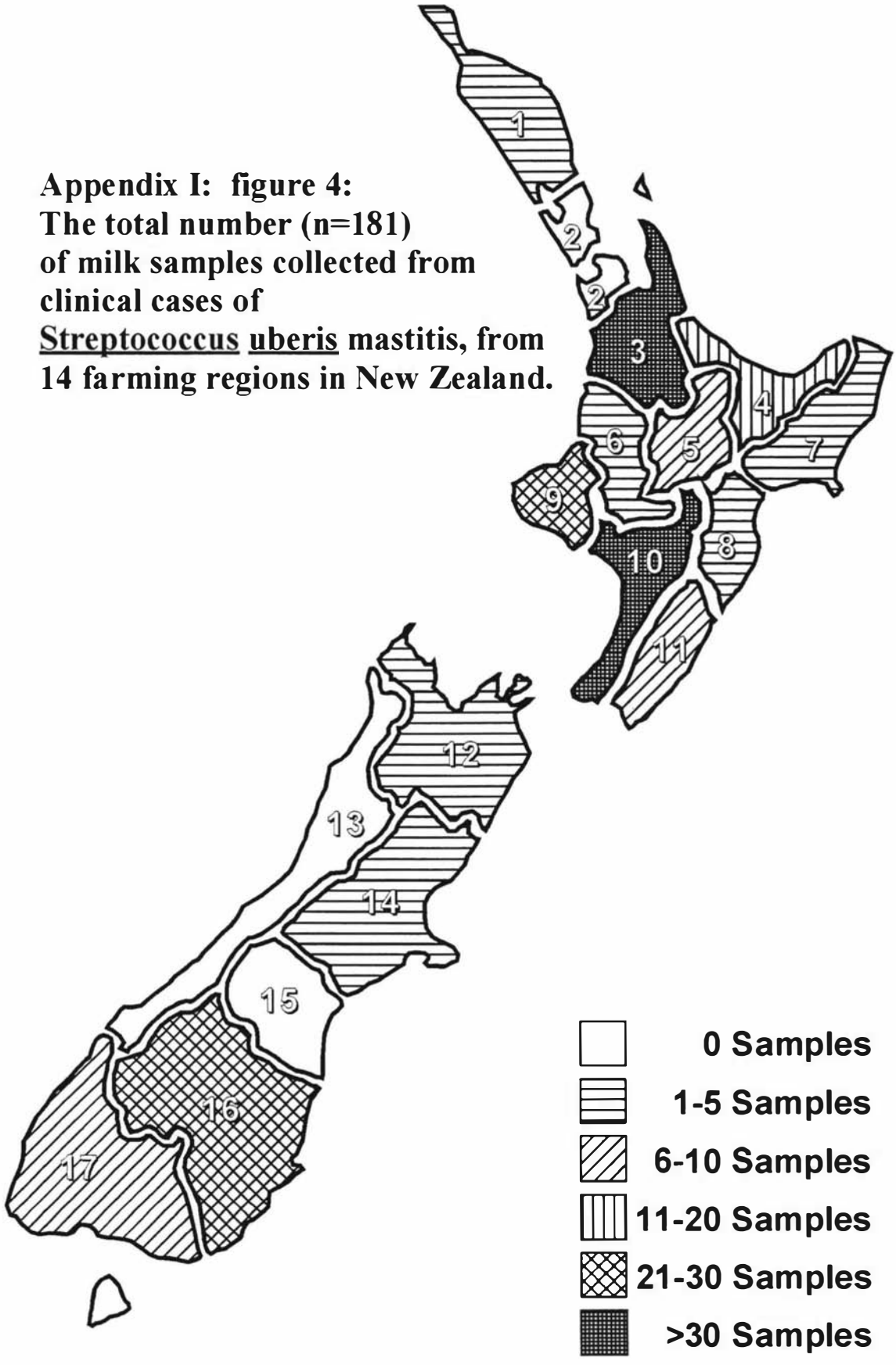
Appendix I: figure 2:
The total number (n=342) of milk samples collected from both subclinical and clinical cases of Streptococcus uberis mastitis, from 15 farming regions in New Zealand.



Appendix I: figure 3:
The total number (n=161) of milk samples collected from subclinical cases of Streptococcus uberis mastitis, from 5 farming regions in New Zealand.



Appendix I: figure 4:
The total number (n=181) of milk samples collected from clinical cases of Streptococcus uberis mastitis, from 14 farming regions in New Zealand.



Appendix II: Media used for pulsed-field electrophoresis

T.E. pH 8.0: (for 100ml)

10 mM TRIS Cl pH 8.0	1 ml – 1 M TRIS Cl pH 8.0
1 mM di-Na^+ EDTA pH 8.0	200:1 - 0.5 M di-Na^+ EDTA pH 8.0
milliequivalent (MQ) water	up to 100 ml

PETT IV Buffer: (for 100ml)

1 M NaCl	20 ml – 5 M NaCl
10 mM TRIS Cl pH 8.0	1 ml – 1 M TRIS Cl pH 8.0
10 mM di-Na^+ EDTA pH 8.0	2 ml - 0.5 M di-Na^+ EDTA pH 8.0
MQ water	up to 100 ml

Lysis Buffer: (for 100ml)

1 M NaCl	20 ml – 5 M NaCl
10 mM TRIS Cl pH 8.0	1 ml – 1 M TRIS Cl pH 8.0
100 mM di-Na^+ EDTA pH 8.0	20 ml - 0.5M di-Na^+ EDTA pH 8.0
0.5% Sarkosyl (N-lauroyl sarcosine)	500 mg Sarkosyl
0.2% Na-deoxycholate	200 mg Na-deoxycholate

just prior to using buffer, 1mg/1ml of lysozyme was added

Urea - ESP Buffer: (for 200ml)

6M urea	72g urea
50 mM TRIS pH 8.0	10 ml of 1M TRIS pH 8.0
50 mM di-Na^+ EDTA pH 8.0	20 ml - 0.5M di-Na^+ EDTA pH 8.0
MQ water	up to 200 ml
Just prior to use add:	
SLS (Sodium lauroyl sarcosine) to 1%	100 mg/10 ml
Na^+ deoxycholate to 0.2%	20 mg/10 ml
Proteinase K to 0.5 mg/ml	5 mg/10 ml

Restriction Buffer: for 20 plugs)

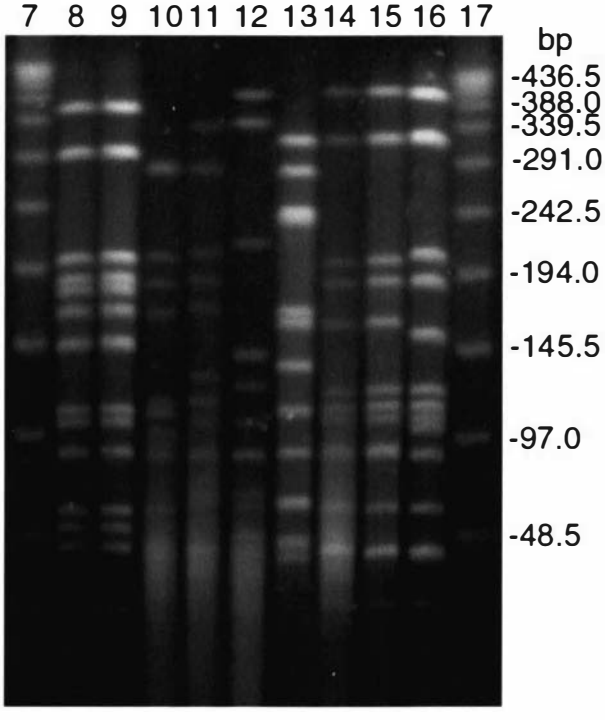
12:1 of 10x NE Buffer 4	240:l/20 plugs
1:1 bovine serum albumin (BSA) @ 10 mg/ml	20:l/20 plugs
87:1 of MQ water	1740:l/20 plugs

Cutting buffer: for 20 plugs)

8:1 of 10x NE Buffer 4	160:l/20 plugs
0.8:1 bovine serum albumin (BSA) @ 10 mg/ml	16:l/20 plugs
72:1 of MQ water	1600:l/20 plugs
15 units (0.75:1) of Sma I	15:l/20 plugs

Appendix III: Legend for figure 1: Photograph of a PFGE gel containing isolates which were incubated for 16 and one for 24 hours at 37 °C.

Appendix III: figure 1:
Photograph of a PFGE gel containing isolates which were incubated for 16 and 24 hours at 37°C.



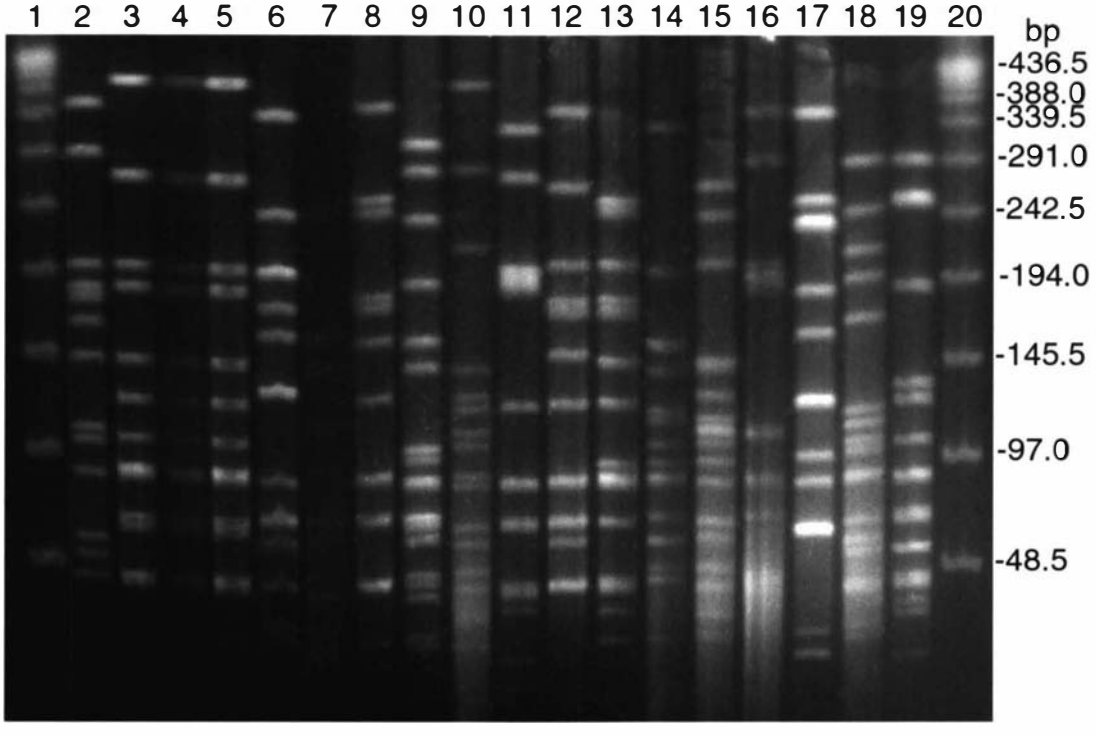
Appendix III: Index for Figure 1: Photograph of a PFGE gel containing isolates which were incubated for 16 and 24 hours at 37°C.

Lane # (from right)	Index #	Farm	Cow ID	District*	Sample Date	Infection
1	(13) 427[a]	B	34	Manawatu	31.10.96	Subclinical
2	(13) 427[b]	B	34	Manawatu	31.10.96	Subclinical
3	70	E	132	Manawatu	23.05.96	Clinical
4	106	A	43	Horowhenua	29.07.96	Subclinical
5	117	D	65	Manawatu	08.09.96	Subclinical
6	139	A	1	Horowhenua	14.09.96	Subclinical
7	426	B	148	Manawatu	31.10.96	Subclinical
8	429	B	148	Manawatu	31.10.96	Subclinical
9	430	B	193	Manawatu	31.10.96	Subclinical

a = 24 hour incubation
b = 16 hour incubation
*** all in the Wellington region**

Appendix III: Legend for figure 2: Photograph of a PFGE gel containing isolates from different regions in New Zealand.

Appendix III: figure 2:
Photograph of a PFGE gel containing isolates from different regions in New Zealand.



Appendix III: Index for Figure 2: Photograph of a PFGE gel containing isolates from different regions in New Zealand.

Lane # (from right)	Index #	Farm	Cow ID	District	Sample Date	Infection
1	Marker					
2	(13) 427	B	34	Manawatu[1]	31.10.96	Subclinical
3	583	J	176	Gore[2]	25.09.97	Clinical
4	584	J	176/2	Gore[2]	25.09.97	Clinical
5	586	J	176/3	Gore[2]	25.09.97	Clinical
6	593	J	079	Clutha[3]	29.09.97	Clinical
7	546	K	90	Franklin District[4]	01.09.97	Subclinical
8	548	L	1	Manawatu[1]	22.09.97	Clinical
9	549	M	118	Manawatu[1]	29.09.97	Clinical
10	550	N	252	South Island[3]	30.09.97	Clinical
11	554	N	9	South Island[3]	30.09.97	Clinical
12	555	N	15	South Island[3]	30.09.97	Clinical
13	557	N	77	South Island[3]	30.09.97	Clinical
14	558	N	36	South Island[3]	30.09.97	Clinical
15	560	N	39	South Island[3]	30.09.97	Clinical
16	561	O	3	Far North[5]	29.09.97	Clinical
17	580	P	81	Upper Hutt[1]	30.09.97	Clinical
18	581	P	42	Horowhenua[1]	30.09.97	Clinical
19	650	Q	99	Whakatane[6]	23.10.97	Clinical
20	Marker					

1 = Wellington region
2 = Southland
3 = Otago
4 = Central Auckland
5 = Northland
6 = Bay of Plenty

Appendix III: Legend for figure 3: Photograph of a PFGE gel containing 18 isolates from Farm #3 and two base pair markers.

Appendix III: figure 3:
Photograph of a PFGE gel containing 18 isolates from farm #3.