The effect of external teat seals on mastitis incidence during the dry period under experimental challenge with *Streptococcus uberis*

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To

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ABSTRACT

Despite decades of research and steady progress in control, mastitis remains one of the most economically important diseases of dairy cattle. The two main periods of high susceptibility to mastitis during the dry period are the first two or three weeks after drying-off and two weeks pre-calving. Dry-cow therapy (DCT), consisting of the use of long-acting antibiotics after the last milking of lactation, has been practiced since the 1960s in order to eliminate existing intramammary infections and prevent new intramammary infections (IMI) during the dry period. The extensive use of antibiotics to treat and control mastitis has possible consequences for human health through an increased threat of emergence of antibiotic-resistant microorganisms that may enter the food chain. Non-antibiotic formulations for the treatment and prevention of mastitis have the potential to reduce the need for antibiotics. The objective of this study was to evaluate the effects of an external teat seal based on tetrahydrofuran (DryFlex (DF)) and a commercially available wound sealant for humans based on 2-octylcyanoacrylate (Band-Aid (BA)) in protecting against mastitis caused by experimental challenge with *Streptococcus uberis* after dry-off, under dairy farming conditions in New Zealand. Cows (175) with a somatic cell count (SCC) of less than 200,000 SCC/ml, four functional quarters, and no signs of clinical mastitis or teat abnormalities were enrolled at dry-off. Single-quarter milk samples were taken for bacteriological culture, four days and one day before dry-off and again on two occasions within four days after calving. After the last milking before dry-off, 88 cows received DF and 87 cows received BA, with pairs of the contra-lateral quarters left front/right back (LF-RB) and right front/left back (RF-LB) assigned to the treatment or the untreated-control groups. All cows were challenged by experimental exposures by dipping all quarters in a bacterial broth of

Streptococcus uberis (1.27 x 10^8 cfu/ml and 1.02 x 10^8 cfu/ml for the first and second experimental exposures, respectively), two and four days after dry-off. Subsequently, assessment for clinical mastitis was performed daily on all quarters. Individual quarters were observed and palpated for the presence of clinical signs consistent with mastitis, i.e., heat, swelling, a painful udder and if required, by an examination for the presence of clots and flakes in the secretions. Following bacterial challenge with *Strep. uberis*, in the DF group, clinical mastitis developed in 35 of the treated quarters and 83 of the untreated quarters. The corresponding data for the BA group are 67 of the treated quarters and 64 of the untreated quarters. The incidence of mastitis was greatest between days 6 and 11 after dry-off for both groups. The risk of mastitis in the DF group was higher in control quarters than in the quarters treated with DF (RR: 2.37, 95% CI 1.70 - 3.32, P = 0.000). There was no significant difference between the treated and untreated quarters in the BA group in the number of new clinical mastitis acquired during the dry period (RR: 0.96, 95% CI: 0.73, 1.25, P = 0.74). *Strep. uberis* was the most prevalent bacterium on days 6-7 and from day 9 onwards, and it was detected in 160 (95%) single-quarter milk samples. The use of pulsed-field gel electrophoresis (PFGE) in a subset of isolates showed that 11 of 19 of the intramammary infections found between six and eleven days after dry-off (peak of infection) were caused by the challenge strain. DryFlex was visible on 50% of the teat-ends for four days. The average number of days for which the teat-ends remained covered with DF was 5.2 for all quarters. The experimental challenge model developed and used in this study appeared to be successful in generating intramammary infections during the dry period of dairy cows and proved a robust test of teat seals. The application of DryFlex at the end of lactation was beneficial in reducing mastitis in dairy cows after dry-off. The octylcyanoacrylate used in the

present study showed no benefit in reducing the incidence of mastitis after dry-off when compared with quarters left untreated. It would appear from the results of this experiment that a teat seal of the DryFlex type would have a role to play in the prevention of mastitis during the early dry period. However, further work is required to improve the persistence of DryFlex on teats of non-lactating dairy cows.

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1. INTRODUCTION

Mastitis is an inflammation of the mammary gland characterized by an increase in the number of somatic cells in the milk and by pathological changes in mammary tissue along with physical, chemical and microbiological changes (Auldist and Hubble, 1998). This disease results from failure in the relationship between three factors: host resistance, mastitis causing bacteria and the environment (Pyörälä, 2002).

The causes of mastitis can be mechanical trauma, chemical injury, or intramammary infections (IMI). However, in the dairy cow, mastitis is mostly caused by bacterial infections.

Depending on the severity of the inflammation, mastitis can be clinically evident or subclinical. The signs of clinical mastitis are swelling, pain, redness and heat of the affected quarters and changes in the composition and the appearance of the milk. The most common form is, however, subclinical mastitis. Although there are neither gross inflammation of the udder nor gross changes in the milk, subclinical mastitis may lead to a significant decrease in milk production and deterioration of milk quality (Auldist and Hubble, 1998).

1.1. The importance of mastitis

Despite decades of research and steady progress in control, mastitis remains one of the most economically important diseases of dairy cattle. There are many costs

associated with clinical and subclinical mastitis. These costs include loss of milk production, milk that has to be discarded after antibiotic treatment, veterinary, labour and replacement costs, plus the cost of lost milk quality premiums or penalties due to increased somatic cell counts (SCC).

With respect to mastitis, the SCC is the most common measurement of milk quality and udder health (Harmon, 1994). The major factor affecting SCC at the herd and cow level is the presence of intramammary infections (Harmon, 1994). The level of infection will affect the annual cost of mastitis in a New Zealand herd (Anon., 2006). Although it is difficult to estimate the costs associated with clinical mastitis as has been highlighted by Petrovski *et al.* (2006), a herd of 200 cows with a SCC of 250,000 cells/ml is likely to be losing about $30 per cow per year on average (Anon., 2006).

Mastitis also causes several changes in the composition of milk, including increases in SCC, which affect the quality and value of the milk, as well as the quality of milk products. Mastitis is associated with a decrease in the concentrations of lactose, α-lactalbumin and fat in milk due to the reduced synthetic activity of the mammary tissue (Harmon, 1994). The concentration of casein, the major milk protein of high nutritional quality, also declines (Kitchen, 1981), but the concentration of whey proteins increases (Harmon, 1994) and this adversely impacts dairy products by reducing cheese yield, negatively impacting flavour and therefore reducing quality. In addition, plasmin and enzymes derived from somatic cells can cause extensive proteolysis of casein in the udder before milk removal (Harmon, 1994). As a result of changes in vascular permeability, increased quantities of serum albumin,

immunoglobulins, transferrin and other serum proteins pass into milk (Kitchen, 1981). Sodium and chloride concentrations are elevated in milk from cows with mastitis because of their passage from blood into milk during mastitis (Harmon, 1994). However, the concentration of potassium, normally the predominant mineral in milk, declines because of paracellular passage out of the alveolar lumen between damaged epithelial cells (Harmon, 1994).

For all of these reasons, it is very important to prevent mastitis. Several strategies have been developed to control mastitis, which include: dry cow therapy (DCT), internal teat seals, immunization regimes, and external teat seals. Although all these strategies will be later described in Section 2.5, it can be said that the extensive use of antibiotics to treat and control mastitis has possible consequences for human health through an increased threat of emergence of antibiotic-resistant microorganisms that may enter the food chain (White and McDermott, 2001). Non-antibiotic formulations for the treatment and prevention of mastitis have the potential to reduce the need for antibiotics. However, since internal teat seals have no antibiotic, unintentional microorganism contamination during infusion, leading to mastitis, is a major problem that may be encountered if inadequate aseptic measures are taken. And although mastitis vaccine technologies have improved, these vaccines are not widely used in programs for mastitis control (Sordillo *et al.*, 1997). Therefore, the aim of the present study was to evaluate the effects of external teat seals in protecting against mastitis caused by experimental challenge with *Streptococcus uberis* after dry-off, under dairy farming conditions in New Zealand.

In this study, *Strep. uberis* was selected as the challenge bacterium because it is the most important environmental pathogen causing mastitis during the dry period in New Zealand (Williamson *et al.*, 1995), as described below, and because it is relatively susceptible to treatment.

1.2. Microorganisms that cause mastitis

Many microorganisms have been shown to cause mastitis; however, most of the economic losses in dairy farms are associated with species of staphylococci, streptococci and coliforms.

Historically, mastitis pathogens are classified as either contagious or environmental based on their primary reservoirs within dairy herds that lead to subsequent infection. The contagious pathogens can be considered as organisms adapted to survive within the host and they are capable of establishing sub-clinical infections. The primary source of contagious pathogens is the udder of infected cows, and they are spread from infected quarters to other quarters and cows primarily during the milking process. In contrast, the environmental pathogens are 'opportunistic' invaders of the mammary gland (Bradley, 2002). They are present in the cow's environment and are not adapted to survive within the host. Typically, they `invade', multiply, cause a host immune response leading to mainly clinical mastitis of short duration (Bradley, 2002).

The major contagious pathogens include *Staphylococcus aureus* and *Streptococcus agalactiae*; and the major environmental pathogens are *Streptococcus uberis,*

Streptococcus dysgalactiae and coliforms, such as *Escherichia coli* and *Klebsiella* sp.

Abbreviations of generic names of microorganisms are not covered by the rules of the International Code of Nomenclature of Bacteria (Lapage *et al.*, 1990). This manuscript will follow Chapter 4 (Advisory Notes) of that code, which provides recommendations on abbreviations.

During recent decades, the predominant bacteriological aetiology of mastitis has changed from contagious to environmental microorganisms (Pyörälä, 2002). In recent years in the United Kingdom (UK), *Strep. uberis* has been reported to be more prevalent than the contagious pathogens (Bradley and Green, 2001a). Increased prevalence of mastitis due to *Strep. uberis* has also been reported in Canada, the United States of America (USA) and the Netherlands.

The prevalence and causes of mastitis in New Zealand dairy herds have also experienced marked changes during the last 50 years (McDougall, 2002). While in 1948 the major pathogen causing both subclinical and clinical mastitis was *Strep. agalactiae* (Roach, 1948), by the mid 1960s *Staph. aureus* was the major pathogen (Elliot *et al.*, 1976). However, now *Strep. uberis* and coagulase-negative staphylococci (CNS) are the most prevalent pathogens causing both subclinical and clinical mastitis (Williamson *et al.*, 1995; McDougall, 1998; Woolford *et al.*, 1998). Infections caused by *E. coli* (McDougall, 2002) and other enterobacteria and Gram-negative bacteria generally have a low incidence in this country since cows in New Zealand are kept on pasture rather than housed as in other countries.

There is a high prevalence of *Strep. uberis*, but its epidemiology is not completely understood (Pullinger *et al.*, 2006). Research has demonstrated that *Strep. uberis* has the ability to resist phagocytosis (Thomas *et al.*, 1994) and intracellular killing by leukocytes (Leigh *et al.*, 1990), thus evading the immune system.

2. LITERATURE REVIEW

2.1. New Zealand dairy systems

The temperate climate that predominates in New Zealand allows pasture to grow and cows to graze outside throughout the whole year. Therefore, dairy systems in this country are based mainly on pasture, which is the cheapest source of feed available and should be fully utilised to maximise efficiency. Less than 10% of the world's total milk supply is produced in grazing systems, such as the one most commonly used in New Zealand (Holmes *et al.*, 2002).

Pasture growth is influenced by seasonal weather patterns and the resulting availability of pasture fluctuates throughout the year. The timing of calving can be manipulated in order to match the feed requirements of the herd (feed demand) with the pasture growth curve (feed supply). The objective is to have high feed demand in spring, when the pasture growth is at its peak and has the highest quality and low feed demand during winter when pasture growth rate is low. Since the period of peak pasture growth occurs at approximately the same time each year, calving must occur at 365-day intervals, requiring cows to conceive within 83 days of calving. Thus, in New Zealand, the majority of dairy herds calve seasonally in spring, resulting in cows being dried-off in autumn, with no lactation for a period of 2 to 4 months. The transmission of pathogens such as *Staph. aureus*, *Strep. agalactiae* and *Strep. dysgalactiae* is restricted because cows are not milked during this period (McDougall, 2002).

On New Zealand dairy farms, a large number of cows are milked per person; therefore, the disinfection of teats is generally achieved by spraying, which is a time-saving approach, as opposed to dipping, as is practiced in other countries (McDougall, 2002). When done appropriately, spraying of teats has been shown to be as effective as dipping of teats in disinfectant (Pankey and Watts, 1983). But if teat spraying is not used, or there is an inadequate teat surface coverage after careless spraying of teats, there could be some limitations to the prevention of mastitis during lactation. In addition, most New Zealand dairy farmers do not perform a pre-milking wash and foremilk inspection. Thus, mastitis detection on many farms is done through observation of the udder and its quarters and the monitoring of SCC in the herd's bulk milk, measured for every milk consignment by the dairy company, as well as observing the milkline filter for evidence of fibrin clots.

The New Zealand average bulk tank somatic cell count (BTSCC) is lower than the BTSCC in much of Europe and United States of America (Franks, 2001). This could be a result of the influence of the coordinated approach of the SAMM plan, together with the imposition of penalties for BTSCC >400,000 cells/ml by most dairy companies and the milk quality testing of every milk consignment (Franks, 2001).

2.2. Dry period: time of susceptibility to mastitis

The dry period is a time of anatomical and physiological changes in the mammary gland. In the early 1950s the dry period was recognised as a time of high susceptibility for new intramammary infections in dairy cattle (Neave *et al.*, 1950).

The two main periods of high susceptibility to mastitis during the dry period are the first two or three weeks after drying-off (early involution) and two weeks pre-calving (colostrogenesis) (Eberhart, 1986; Green *et al.*, 2002).

There are several reasons why early involution is a high-risk period for bacterial infection. Firstly, the occurrence of increased intramammary pressure after cessation of milking results in leakage of milk from the glands and predisposes to bacterial penetration (Cousins *et al.*, 1980; Breau and Oliver, 1986; Bushe and Oliver, 1987). In addition, the increased volume of milk in the gland has lower concentrations of lactoferrin, immunoglobulins (Ig) and phagocytic cells, all of which are protective factors to the mammary gland (Bushe and Oliver, 1987).

Another cause for increased susceptibility to mastitis during early involution is that since milking is stopped and milk is no longer being removed, the effect of flushing bacteria out of the gland has ceased. At this time, there is also a cessation of teat hygiene practices that reduce the risk of new infections (Green *et al.*, 2002).

During the prepartum period, the reasons for high susceptibility to mastitis are the increased intramammary pressure as colostrum is produced (that may lead to leaking of colostrum), a decreased number of circulating neutrophils, a decreased capacity of neutrophils to kill bacteria and a delayed inflammatory response (Meglia *et al.*, 2001).

Quarters infected with microorganisms classed as major pathogens at dry-off have a higher risk of developing a new IMI during the dry period (Eberhart, 1986). Many

infections that occur during the early dry period persist throughout the dry period and can be a major cause of clinical mastitis during the following lactation (Bushe and Oliver, 1987) and have a significant impact on milk production (Dingwell *et al.*, 2003). Research has shown that 50 to 55% of dry period subclinical mastitis will become clinical after calving (Todhunter *et al.*, 1995; Williamson *et al.*, 1995). Consequently, it is very important to prevent mastitis during the dry period.

Contrasting results have been found concerning the protective effect of infection by minor pathogens against IMI caused by major pathogens. Some studies have shown that quarters infected with minor pathogens are at lower risk of developing a new IMI caused by a major pathogen (Rainard and Poutrel, 1988; Lam *et al.*, 1997). However, when specific microorganisms were considered, other studies have contradicted those findings (Hogan *et al.*, 1988; Berry and Hillerton, 2002b).

2.3. Factors affecting new intramammary infections

2.3.1. Teat canal

The first line of defence against bacterial entry into the mammary gland is considered to be a physical barrier, i.e. the teat canal. It is lined with keratin, a waxy substance that is derived from the stratified squamous epithelium and is crucial to maintain the barrier function of the teat canal because it has antimicrobial properties (Sordillo, 2005). As a result of the structure of keratin, microorganisms are adsorbed in this substance and therefore, they cannot migrate further into the mammary gland (Sordillo *et al.*, 1997).

The formation of the teat canal keratin plug at the appropriate time after dry-off establishes a natural protective mechanism against mastitis infections. There is however little research about the proportion of quarters that form a keratin plug in the teat canal and the length of time that it takes for the plug to be formed (Dingwell *et al.*, 2003). One of the few studies to assess the status of the teat canal keratin plug was conducted in New Zealand (Williamson *et al.*, 1995). During the dry period, the formation of an effective teat canal keratin plug was assessed manually every two weeks. This involved applying light pressure to the teat sinus using a gentle milking action with the thumb and index finger. Closed teats were those from which no fluid could be expressed when the milking action was applied to them. It was found that by 30-40 days after drying-off, there was a 50% decline in the incidence of open teats. From 50 to 90 days after drying-off, 3 to 5% of teats were still open, with a higher proportion of open teats in hind quarters than in front quarters. In addition, it was observed that quarters that had received DCT formed the keratin plug earlier than quarters that had not received DCT. This may mean that the physical sealing of the teat canal after dry-off was facilitated somehow by the DCT; however, the mechanism responsible for this is not clearly understood (Williamson *et al.*, 1995). A proposed explanation is that bacterial enzymes within the teat canal may slow the accumulation of a solid mass of desquamated cells (Williamson *et al.*, 1995). Killing of the bacteria that produce those enzymes by the use of DCT may have increased the rate of teat canal closure (Williamson *et al.*, 1995).

Another study was performed in USA with the objective of associating cow and quarter-level factors during late lactation on new IMI during the dry period (Dingwell *et al.*, 2004). Teat-end scores and the formation of the teat canal keratin

plug were evaluated once a week. The rate of teat closure influenced new IMI during the dry period. Quarters that closed during the dry period were 1.8 times less likely to develop a new IMI. The study also reported a similar linear decline in the percent of open teats to that observed in the New Zealand study, but there was no significant difference in the rate of closure between hind and front quarters (Dingwell *et al.*, 2004). A possible explanation for this difference could be differences in the criteria used define teat canal closure in both studies. The impact of DCT could not be assessed in this second study since all quarters received DCT (Dingwell *et al.*, 2004).

The length and diameter of the teat canal and weight of keratin occluding the lumen of the teat canal can affect resistance to mastitis (Lacy-Hulbert and Hillerton, 1995). Short and wide canals allow faster rates of milk flow, but they may have less resistance to mastitis. In two experiments it was found that a negative relationship existed between the length of the teat canal and the probability of infection with *Strep. uberis* and *Strep. agalactiae* under normal milking conditions (Lacy-Hulbert and Hillerton, 1995).

2.3.2. Teat-end integrity

The integrity of the teat-end with respect to lesions and hyperkeratosis has been evaluated as a factor affecting susceptibility to IMI infections (Dingwell *et al.*, 2003). The results of the study by Dingwell *et al.* (2004) showed that a teat-end crack was an important risk factor for new IMI in the dry period. Quarters with a cracked teat-end were 1.7 times more likely to develop new IMI than quarters that did not have a crack (Dingwell *et al.*, 2004). It is likely that any increase in the surface area of teat-ends through cracks and hyperkeratosis could lead to an increase in the total number

of bacterial pathogens present, which could increase the probability to develop mastitis infections (Dingwell *et al.*, 2003).

2.3.3. Parity of cows

There is a relationship between the parity of a cow at time of dry-off and development of new IMI during the early dry period (Todhunter *et al.*, 1995). Cows that finished their fourth or greater lactation had a higher rate of new streptococcal IMI than cows of lower lactation number and these rates of new IMI were significantly higher during the first half of the dry period (Todhunter *et al.*, 1995). However, parity had no influence on the rate of IMI during the last half of the dry period (Todhunter *et al.*, 1995). Another study showed that cows of first or second lactation had 2.6% of quarters infected after the dry period compared with 23.8% of infected quarters of cows finishing their third or greater lactation (Oliver and Mitchell, 1983). One possible explanation for this is that generally, older cows have a more patent teat canal making them more susceptible to the occurrence of new IMI (Oliver and Mitchell, 1983).

2.3.4. Milk production at time of dry-off and method of milk cessation

Cows producing large quantities of milk before drying-off could be at higher risk of developing an IMI than cows producing less milk, due to the increased intramammary pressure and lower concentrations of lactoferrin, immunoglobulins and phagocytic cells (Bushe and Oliver, 1987). In a study in the UK, it was shown that for every 1 L increase in final milk yield, cows were 1.06 times more likely to acquire a new enterobacterial IMI (Huxley *et al.*, 2002). However, it is possible that the higher risk of becoming infected with a new enterobacterial IMI is a function of

high-yielding dairy cows rather than yield prior to dry-off (Huxley *et al.*, 2002). Therefore, more research needs to be done in order to clarify the relative importance of milk production at drying-off, especially as the genetic potential for milk production of cows continues to increase.

Cessation of lactation can be managed by either abrupt or intermittent dry-off procedures. Abrupt cessation of milking means that cows are milked as usual and the farmer stops milking the cows on the planned day of dry-off. The term intermittent has a wider definition because it can mean, for example, that cows are milked once a day for the week preceding drying-off, or they are milked once a day every other day during the two weeks prior to dry-off (Dingwell *et al.*, 2003). The effect of the method of drying-off on new IMI has been investigated by Bushe and Oliver (1987). In their study, cows milked intermittently and fed only hay produced less milk and that milk contained higher concentrations of lactoferrin (Lf) and IgG compared to milk from cows dried off by abrupt cessation of milking or only milked intermittently before cessation. However, there were few differences in bacterial growth inhibition between mammary secretions from cows milked intermittently and those dried off by abrupt cessation of milking (Bushe and Oliver, 1987). Douglas (1999) carried out an experiment to compare the effect of two drying-off techniques on the prevalence of new intramammary infections. Cows were milked either once daily (OAD) for the last eight days of the lactation or once every other day (EOD) for the same period. It was found that on the last day of lactation, the odds of a quarter being infected with pathogens were 2.4 times higher for cows milked EOD than for cows milked OAD (Douglas, 1999). The SAMM plan recommends that

cows should be dried off abruptly without omitting any day of milking before drying-off (Anon., 2006).

2.3.5. Environment

The bacterial population of mastitis-causing microorganisms on teats during the dry period is a function of natural exposure from the cow's environment together with a cessation of teat hygiene practices and cessation of milking (Eberhart, 1986). Continued exposure to new bacteria from the environment is an important factor influencing the occurrence of new IMI during the dry period. Thus, proper maintenance of the cow's environment in the dry period, in order to minimize exposure to bacteria, will reduce infection rates (Eberhart, 1986). In countries where cows are kept indoors, it is highly recommended that the calving area is kept as dry and clean as possible with the bedding material changed often (Dingwell *et al.*, 2003). In New Zealand, where cows graze pastures all year round, it is recommended that cows should calve on clean and fresh pastures.

2.4. Mammary defence mechanisms against mastitis pathogens

The incidence of mastitis increases when defence mechanisms of the mammary gland are impaired. Natural disease resistance factors in the mammary gland are innate or non-specific immunity and acquired or specific immunity (Sordillo, 2005).

Innate immunity is the predominant defence mechanism during the early stages of infection and is mediated by the physical barrier of the teat canal (described in section 2.4.1), macrophages, neutrophils, natural killer cells and by certain soluble factors (Sordillo, 2005).

Specific immunity is mediated by antibody molecules, macrophages and several lymphoid populations. In contrast to innate immunity, specific immunity can be augmented by repeated exposure to a pathogen. Both types of immunity should interact in a highly coordinated way in order to optimally protect the mammary gland from new intramammary infections (Sordillo, 2005).

2.4.1. Cellular immunity against infection

Once bacteria pass the physical barrier of the teat canal, they must overcome the mammary gland's immune system in order to cause infection (Sordillo *et al.*, 1997). The immune cells include leukocytes, which consist of neutrophils, macrophages and lymphocytes. The total number and activity of mammary gland leukocytes play an early and vital role in determining the severity and duration of intramammary infections (Sordillo, 2005). They are responsible for the high SCC that is characteristic of mastitic milk and are associated with many of the changes in milk protein composition that occur during mastitis (Auldist and Hubble, 1998).

Neutrophils are the predominant cell type of leukocytes in mammary tissues and mammary secretions during early inflammation (Sordillo *et al.*, 1989). The population of neutrophils is relatively low in a healthy mammary gland ($<10^5$ cells/ml) but they can be more than 90% ($>10^6$ cells/ml) of the total mammary leukocyte population during an intramammary infection (Sordillo, 2005). Since polymorphonuclear neutrophils (PMN) phagocytose bacteria and kill organisms intracellularly, they are the major defence mechanism of the mammary gland in addition to teat keratin (Nickerson *et al.*, 1987; Paulrud, 2005). It is thought that during an intramammary infection, PMN migrate across the teat duct lining into the

lumen (Paulrud, 2005). During the peripartum period, the phagocytic activity of neutrophils is reduced (Sordillo *et al.*, 1997).

Macrophages are the predominant cell type in milk of healthy, lactating mammary glands but are a minority during inflammation. Macrophages phagocytose bacteria and destroy them with proteases and reactive oxygen species. Although the population of macrophages is highest during the last week of gestation, the phagocytic capacity of macrophages is reduced during that time (Sordillo, 2005).

Lymphocytes recognize antigens via specific membrane receptors which define the immunological characteristics of specificity, diversity, memory and self/non-self recognition (Sordillo, 2005). There are two general types of lymphocytes: B cells and T cells and they differ in function and protein products (Sordillo, 2005).

2.4.2. Soluble defences against infection

The primary soluble effectors of the specific immune response are immunoglobulins, which are antibodies produced by antigen-activated B-lymphocytes (Sordillo, 2005). They mainly penetrate into the mammary gland but they are also locally formed (Kehrli and Harp, 2001). There are four Ig isotypes that influence mammary gland defence against bacteria that cause mastitis and they are IgG_1, IgG_2, IgA and IgM (Sordillo *et al.*, 1997). In healthy udders, the concentration of Ig is low during lactation but gradually increases during the dry period (Sordillo *et al.*, 1997). In infected mammary glands, stage of lactation and infection status influence the concentration of each Ig class in mammary secretion (Sordillo *et al.*, 1997). The Ig classes, IgG_1, IgG_2 and IgM can enhance phagocytosis by neutrophils and

macrophages (Sordillo *et al.*, 1997), while IgA seems to contribute to agglutination, preventing bacterial colonization and to toxin neutralization (Kehrli and Harp, 2001).

The mammary gland also has non-specific bacteriostatic factors that act independently and together with Ig and cellular factors to give protection to the mammary gland. These factors include lysozyme, lactoferrin, complement, cytokines and the lactoperoxidase-thiocyanate-hydrogen peroxide system (Sordillo *et al.*, 1997). However, lysozyme, complement and the lactoperoxide system will not be described in this manuscript since they play only a minor role in the protection of the mammary gland against microorganisms causing mastitis.

Lactoferrin is an iron-binding glycoprotein present in bovine milk (Fang *et al.*, 2000). It is produced by epithelial cells and leukocytes and in the presence of bicarbonate, it fixes free ferric ions that are in milk (Sordillo *et al.*, 1997). Therefore, it has a bacteriostatic role because it prevents growth of bacteria that require iron (Sordillo *et al.*, 1997), such as aerobic bacteria (Pyörälä, 2002). However, streptococci including *Strep. uberis* are more resistant to the bacteriostatic effects of Lf than Gram-negative bacteria. This may be due to the low iron requirements of these bacteria. In the presence of citrate, the bacteriostatic role of Lf can be stopped (Sordillo *et al.*, 1997). Citrate is a buffer produced by epithelial cells that chelates iron into a form that is readily available for use by bacteria (Sordillo *et al.*, 1997). The concentrations of citrate and Lf change during mammary involution, establishment of lactation and mastitis infections (Nonnecke and Smith, 1984). During lactation in a healthy mammary gland the concentration of lactoferrin is lowest while that of citrate is highest. That might be the reason why it is unlikely that

lactoferrin would have a strong effect during lactation (Pyörälä, 2002). The concentration of Lf increases during the dry period and in mastitic glands (Sordillo *et al.*, 1997).

Cytokines are proteins produced by a variety of immune and non-immune cells (Sordillo *et al.*, 1997). There are numerous cytokines and the major groups are interleukin (IL), colony-stimulating factor (CSF), interferons (IFN) and tumor necrosis factor (TNF) (Sordillo *et al.*, 1997). Interleukin-2 is the most widely described of bovine cytokines (Sordillo, 2005). It is responsible for the growth and differentiation of effector and memory T lymphocytes and the activation of B lymphocytes (Sordillo, 2005). CSF is required for the proliferation and differentiation of macrophages and T cells (Sordillo, 2005). IFN-γ elicits functional changes on lymphocytes and phagocytic cells in the mammary gland (Sordillo, 2005). TNF-α is a mediator of endotoxic shock during peracute coliform mastitis (Sordillo *et al.*, 1997).

2.5. Management strategies to prevent new intramammary infections

2.5.1. Seasonal Approach to Managing Mastitis (SAMM) plan in NZ

The SAMM plan is a six-stage prevention programme first developed in 1993 by the Dairying Research Corporation together with the Livestock Improvement Corporation (LIC). It was implemented as a means to reduce the incidence of mastitis and to improve milk quality nationwide, by providing farmers with consistent recommended management strategies to reduce the incidence and prevalence of mastitis in their herds. It is now managed by the National Mastitis

Advisory Committee via Dexcel and the information, in the form of a booklet, is distributed to dairy farmers through dairy companies (Anon., 2006).

This plan follows the principles of the Five-Point Plan developed in United Kingdom in the 1960s, where non-seasonal calving predominates. The main points of the Five-Point Plan are: prompt treatment of all clinical cases during lactation, use of dry-cow therapy (DCT) for all cows, efficient and effective post-milking teat disinfection, culling of chronically infected cows and checking of milking equipment regularly (Bradley, 2002). These points are included in the SAMM plan, but for New Zealand timing is synchronized with respect to seasonally calving herds managed on pasture. The SAMM plan is organized into a logical schedule based on the six major periods of the dairy season: late lactation, drying-off, dry period, calving period, colostrum and lactation (Anon., 2006). Table 2.1 shows the measures to control mastitis recommended by the SAMM plan, divided in each period of the dairy season.

Research during the late 1980s showed that on average, SCC higher than 120,000 cells/ml and 150,000 cells/ml for heifers and cows, respectively, were reliable indications of the presence of an intramammary infection and those thresholds have now become accepted for use in the SAMM plan (Holdaway, 1990).

2.5.2. Dry-cow therapy

Dry-cow therapy consists of infusing a long-acting antibiotic into the mammary gland via the teat canal of quarters after the last milking of lactation. This method has been practiced since the 1960s in order to eliminate existing IMI and prevent new IMI during the dry period (Dodd *et al.*, 1969).

Table 2.1. Control measures of mastitis for each period of the dairy season recommended by the SAMM plan (adapted from Anon., 2006).

Period of the dairy season	Actions to help control mastitis
Late lactation	• Check records of clinical cases and individual cow SCC • Decide dry cow strategy • Dry off low yielding and high SCC cows early
Drying-off	• Dry-off abruptly (no skip a day milking) • Administer dry cow therapy hygienically • Check quarters of dried-off cows for clinical mastitis
Dry period	• Test milking machine and correct faults • Treat clinical cases and record treatment details • Enrol for herd testing • Review and set up recording system • Before calving starts, plan the mastitis management and treatment choices • Train staff in control and treatment methods
Calving	• Calve on clean pastures • Milk out completely • Minimise suckling
Colostrum	• Run colostrum cows as a separate mob • Check foremilk for clinical signs at each milking • Milk out completely • Test for sub-clinical infections before putting into the main herd • Teat spray
Lactation	• Monitor machine and correct faults • Run treated cons in a separate mob • Milk out thoroughly • Monitor bulk milk cell count • Teat spray every time cows go through the dairy

However, although these antibiotics are effective in preventing new infections in the period when there is the highest rate of occurrence of new infections, antibiotics do not last long enough to prevent infections in the late dry period when there is also a high risk of acquiring new IMI (Bradley and Green, 2001b; Crispie *et al.*, 2004; Young *et al.*, 2005).

Many experiments have evaluated the efficacy of DCT in preventing and curing mastitis in the dry period of dairy cows. It is not within the scope of this thesis to review all the literature available on DCT. Nevertheless, some points of interest are worthy of noting.

DCT is still one of the key measures in mastitis control programmes in many countries (Dingwell *et al.*, 2003). However, different countries have different adoption rates of DCT (Dingwell *et al.*, 2003). Prophylactic use of antibiotics in uninfected quarters could predispose to infection by accidental introduction of pathogens resistant to the antibiotic, by disruption of the teat canal epithelium, or via disruption of the normal intramammary micro-flora (Williamson *et al.*, 1995).

Treatment of IMI at drying-off has more advantages than treatment during lactation because high antibiotic doses can be used safely and the retention time of the antibiotic in the mammary gland is longer resulting in the cure rate being higher than in lactation, and also there is no milk loss due to withholding time (Nickerson *et al.*, 1999).

With the use of intramammary treatment, approximately 70 to 90% of IMI at dry-off are cured by the time of calving (Sol *et al.*, 1994). It was shown that DCT was more effective than no treatment in curing existing *Strep. uberis* infections and reducing the intramammary incidence of *Strep. uberis* in cows, regardless of whether a cow was previously uninfected or infected at dry-off (Williamson *et al.*, 1995).

The cure rate after treatment of quarters infected with *Staph. aureus* at dry-off is less successful than for other microorganisms (Eberhart, 1986) averaging 50% (Browning *et al.*, 1990). Many IMI caused by *Staph. aureus* that are treated at drying-off persist through the dry period, resulting in clinical cases at calving (Nickerson *et al.*, 1999). Possible reasons for the lower cure rate of *Staph. aureus* are: resistance to antibiotics, poor distribution of antibiotics throughout the udder, formation of microabscesses, decreased polymorphonuclear cell phagocytosis and intracellular viability of the organisms (Leslie, 1994). The cure rate of IMI caused by *Staph. aureus* was found to be influenced by the age of the cow, the location of the infected quarter, the SCC of infected quarters and the number of quarters infected with *Staph. aureus* (Sol *et al.*, 1994). New products and therapy regimens are needed to improve cure rates in quarters infected by *Staph. aureus* and treated at dry-off (Nickerson *et al.*, 1999).

Blanket DCT means that all cows in the herd receive antibiotic treatment at the end of lactation. However, this regime has been challenged because of the risk of the emergence of bacteria resistant to antibiotics, an increased risk of antibiotic residues in milk products (Berry and Hillerton, 2002b), and the cost of the antibiotics. Therefore, selective treatment of cows or quarters assumed to be infected, called selective DCT or selective dry quarter treatment (DQT), have been proposed for

certain conditions. For these methods to be implemented, it is necessary to correctly identify infected cows/quarters and cows/quarters at risk. The availability of SCC information at the cow-level in dairy herds assists the use of selective DCT (Robert *et al.*, 2006). However, selection based only on SCC is usually of lower value than bacteriological examinations (Dohoo and Leslie, 1991). Consequently, this may result in treating some false positive (uninfected) cows and leaving untreated some false negative (infected) cows (Robert *et al.*, 2006). Hence, use of selective DCT may lead to a higher prevalence of IMI in the following lactation than blanket DCT (McDougall, 2003). On the other hand, the use of bacteriology under field conditions increases costs and creates the need to sample aseptically and to transport samples (Huxley *et al.*, 2002; Robert *et al.*, 2006), which is probably physically and financially unfeasible in large herds.

Selective DQT, in comparison with selective DCT, was found to have a higher risk of new IMI (Robert *et al.*, 2006). In addition, selective DQT requires a method to identify the infected quarters and in practical conditions, the availability of information of SCC at the cow-level (instead of quarter-level) makes the implementation of DCT easier (Robert *et al.*, 2006).

Under field conditions, teats are less carefully disinfected when infusing DCT than under research studies (Robert *et al.*, 2006). Data from 158 herds across the Waikato region of New Zealand showed that 15% of the farmers surveyed used no teat disinfection before infusion of DCT (McDougall, 2003). This increases the risk of introducing a new infection into a non-infected quarter.

2.5.3. Internal teat seals

In addition to the financial implications of mastitis, the importance of this disease and its treatment in public health should not be ignored (Bradley, 2002). The extensive use of antibiotics to treat and control mastitis has possible consequences for human health through an increased threat of emergence of antibiotic-resistant microorganisms that may enter the food chain (White and McDermott, 2001). Non-antibiotic formulations for the treatment and prevention of mastitis have the potential to reduce the need for antibiotics.

Research on an internal teat seal, infused directly into the teat canal after the last milking, began in Ireland in 1972 (Meaney, 1976). The teat seal consisted of a heavy inorganic salt (bismuth subnitrate) in a paraffin/wax base without antibiotic (Meaney, 1977). Only 3.5% of quarters with teat seal alone became infected in comparison to 32% of quarters with DCT alone (Meaney, 1977). When the teat seal with antibiotic was compared with DCT alone, 5.8% of quarters became infected versus 32.4% of quarters that received DCT (Meaney, 1977). X-rays showed that the teat seal remained in position for 3 to 4 weeks in the teat ducts and sinuses (Meaney, 1977).

Later, this product was reformulated in New Zealand resulting in double the concentration of bismuth subnitrate and was tested under natural field conditions in this country. In quarters infused with the teat seal at dry-off, the overall incidence of new IMI over the dry period and including calving, pooled across herds and pathogens, was significantly lower than that of untreated quarters (negative control). It had the same level of protection as long-lasting antibiotic quarters (positive control) and quarters treated with antibiotic and the teat seal (Woolford *et al.*, 1998)

(Table 2.2). The teat seal persisted as a physical barrier in the teat canal over 100 days of the dry period (Woolford *et al.*, 1998).

Table 2.2. Number of new intramammary infections (IMI) over the dry period and at calving in quarters of untreated cows (negative control) and of cows treated with a dry cow antibiotic (positive control), teat seal or antibiotic plus teat seal[a] (Adapted from Woolford *et al.*, 1998)

Experimental group	Negative control	Positive control	Teat seal	Antibiotic + teat seal
Total quarters treated	528	528	505	505
New dry period clinical IMI	18	2*	1*	2*
New IMI at calving	67	12*	12*	8*
Total new IMI	85	14*	13*	10*
Overall new IMI rate (% quarters)	16.1	2.7*	2.5*	2.0*

[a]Numbers within rows marked with * differed significantly ($p<0.01$) between treatments.

Other studies have shown that the application of the internal teat seal at dry-off resulted in significantly fewer new IMI at calving than occurred in untreated cows (Berry and Hillerton, 2002a), or in cows that received antibiotic alone (Huxley *et al.*, 2002; Godden *et al.*, 2003), or when used as an adjunct to DCT in comparison to DCT alone (Godden *et al.*, 2003; Sanford *et al.*, 2006). However, it appears to be more effective in animals that have low SCC at the time of application (Berry and Hillerton, 2002a). Thus, farmers should appropriately select cows to receive the internal teat seal.

One advantage of the internal teat seal is that it is easily hand-stripped out of the quarter after calving and it has no antimicrobial properties or residue issues.

However, since this product has no antibiotic, unintentional microorganism contamination during infusion, leading to mastitis, is a major problem that may be encountered if inadequate aseptic measures are taken.

The benefits of internal teat seal formulations may be enhanced by using non-antibiotic additives such as bacteriocins (Twomey *et al.*, 2000), which are proteins produced by lactic acid bacteria (Ryan *et al.*, 1998). Lacticin 3147, a type of bacteriocin produced by *Lactococcus lactis*, inhibits a wide range of Gram-positive bacteria including some mastitis-causing microorganisms such as *Staphylococcus* and *Streptococcus* species (Ryan *et al.*, 1998). Studies showed the potential for a bacteriocin-based non-antibiotic internal teat seal in preventing mastitis during the non-lactating period (Ryan *et al.*, 1998; Twomey *et al.*, 2000). While the bismuth-based teat seal alone provided an effective physical barrier against infection, the addition of lacticin 3147 improved the product by inhibiting any infectious Gram-positive microorganisms, which may have had the ability to evade the teat seal plug in the teat canal (Ryan *et al.*, 1998). Additionally, it was well tolerated by udder tissue (Ryan *et al.*, 1998).

2.5.4. External teat seals

Application of an external barrier is a less-invasive way of protecting the teat-end during the dry period. Advantages of external teat seals over those applied internally include: a) the formation of a physical barrier at the external teat orifice during the dry period to prevent entry of mastitis infection under natural exposure; b) they are non-invasive and therefore do not destroy the natural keratin plug in the teat canal; c) they are non-irritating and pose no threat to the cow, environment or milk quality;

and d) they are easy to apply. However, their use also presents disadvantages, including: a) they are recommended to be used as an adjunct to antibiotic therapy rather than as a single alternative for mastitis control during the dry period; b) even when proper application process is followed, they only last for an average of 3 to 7 days on the teats (Timms, 2001); c) for full benefit they must be applied at dry-off, then reapplied 10 days pre-calving and the application should be repeated as needed until calving.

Originally, external teat seals were developed for the prevention of mastitis between milkings during lactation (Farnsworth *et al.*, 1980). The teat seal used was an acrylic latex film without germicide applied by dipping immediately after each milking in the same way as a conventional teat dip, and remained intact as a physical barrier until the next milking. A half udder design was used with the right side quarters dipped in the teat seal while the left side quarters served as untreated controls. The objective of the study was to evaluate the efficacy of the teat seal by comparing the incidence of bacterial infections in treated versus untreated quarters. The teat seal reduced by 28%, 33% and 76% the rates of IMI caused by *Staph. aureus*, coagulase-negative staphylococci and coliforms, respectively, but caused no significant reduction in *Strep. agalactiae* and environmental streptococci, and it was non-irritating to the skin (Farnsworth *et al.*, 1980).

A few years later, the efficacy of a latex teat seal was evaluated under field conditions during the dry period of dairy cows (McArthur *et al.*, 1984). One side of the udder was dipped with the teat seal while the other side served as untreated control. Rates of new coliform, staphylococcal and streptococcal infections were

similar in treated quarters and in untreated quarters, suggesting that the teat seal was not effective in preventing infections by those organisms. Another drawback of this product was the poor adhesion and lack of durability. The effectiveness of this product as a protective teat seal was then investigated using an experimental challenge with *E. coli.* The broth was applied on top of the dried film that the teat seal had formed. The challenge organism was recovered from 33% of treated quarters and 42% of control quarters 6 hours after exposure (McArthur *et al.*, 1984).

Later, the addition of a germicide to an acrylic latex teat dip was evaluated in periparturient cows to determine its efficacy in the prevention of new IMI at parturition (Matthews *et al.*, 1988). The day of dry-off, all quarters received DCT. Starting two weeks prior to the expected calving date, the teat seal was applied once daily until parturition. A half udder design was used, the left quarters were treated with the teat seal and the right quarters remained as undipped controls. There was no significant difference in new infections at calving between quarters that had been dipped in the seal with those that had not received the seal (Matthews *et al.*, 1988). The major problem found by these authors was the lack of persistence of the product to maintain a physical barrier on the teat-end for at least 24 h.

Another study evaluated the use of a barrier teat dip containing 0.1% polyvidon iodine on the prevalence of IMI and the incidence of clinical mastitis in periparturient heifers (Edinger *et al.*, 2000). The left front and right hind quarters of the heifers were treated with the teat dip three times weekly, starting from day 260 after the last breeding until calving. The right front and left hind quarters were left as untreated controls. It was found that there was no effect of the barrier teat dip on the

prevalence of IMI at calving and the incidence of clinical mastitis during the first five days of lactation (Edinger *et al.*, 2000). Possible reasons for the lack of positive effect of the teat dip could be that the germicidal activity of the film might have been decreased, being insufficient to prevent infection; the frequency of application might have been insufficient; or that the teat dip did not work against contagious bacteria but against environmental pathogens which were a minor problem in the herd used for the study (Edinger *et al.*, 2000).

An external teat sealt based on tetrahydrofuran was available in USA and Europe but there is little published research on it. Timms *et al.* (1997) evaluated this external teat seal in a natural exposure field trial, with a randomized half udder design with either right or left quarters treated while the others served as untreated controls. All quarters were treated with an intramammary antibiotic on the day of dry-off, followed by one dipping of all teats in the external teat seal group. Ten to fourteen days pre-partum, all treated teats were dipped in the teat seal and redipped as needed until calving (as was recommended by the manufacturer). Quarters treated with the external teat seal plus DCT at dry-off and reapplied pre-calving, resulted in 68, 43 and 47% reduction in environmental streptococci, CNS and total IMI at calving in dairy cows, in comparison with untreated quarters (Timms *et al.*, 1997) (Table 2.3).

A second trial carried out by Timms (2001) was similar to the previous study in terms of dipping with the external teat seal but only half the quarters were treated with DCT. The objective was to evaluate the potential role of the teat seal as an alternative to DCT for the prevention of IMI in the early dry period. This author used four experimental groups: external teat seal at dry-off (ETS), dry cow therapy

(DCT), external teat seal at dry-off plus dry cow therapy (ETS/DCT) and untreated quarters (control). It showed that infection levels after dry-off for control and treated quarters were 13% and 5%, respectively. Infection levels for all groups were low, however all treatments produced significant reductions in IMI compared to untreated quarters (Table 2.4). The external teat seal alone, or when used in combination with intramammary antibiotics, provided an additional reduction in dry-period mastitis in comparison to dry-cow therapy alone, with no significant difference between ETS and ETS/DCT treatments (Table 2.4).

Table 2.3. Number of new intramammary infections (IMI) at calving in quarters treated with the external teat seal plus antibiotic at dry-off and reapplied pre-calving (treated) and untreated quarters (untreated) (Adapted from Timms, 2001)

	Treated	Untreated
Total quarters allocated	246	246
Total quarters without infection	213	184
Environmental streptococci	7[c]	22
Staphylococcus aureus	1	0
Coagulase-negative staphylococci	20[c]	35
Gram-negatives	5	5
Total IMI	33[c]	62

[a,b,c] Significant lower IMI compared to control quarters: [a] $p<0.1$; [b] $p<0.05$; [c] $p<0.025$.

Another study used the same external teat seal as Timms (2001), for three treatment groups: dry cow therapy (DCT), dry cow therapy plus external teat seal at dry-off (DCT/ETSD) and dry cow therapy plus external teat seal pre-calving (DCT/ETSC). There were reductions of 23% and 49% in new IMI in cows treated with DCT and the external teat seal at dry-off and treated with DCT and external teat seal pre-calving, respectively, compared with cows treated with DCT alone. Therefore, it can

be concluded from this study that the application of the external teat seal at dry-off is beneficial, but it is even more favourable when is applied pre-partum (Young *et al.*, 2005).

Table 2.4. Number of non-infected quarters and total intramammary infections (IMI) in control, external teat seal (ETS), dry cow therapy (DCT) and external teat seal plus dry cow therapy at dry-off (ETS/DCT) quarters during the early dry period, without experimental bacterial challenge (Adapted from Timms, 2001)

	Treatment			
	Control	ETS	DCT	ETS/DCT
Total quarters without infection	94	104	105	108
Total IMI	14	5[c]	6[b]	4[c]

[a,b,c] Significant lower IMI compared to control quarters: [a] $p<0.1$; [b] $p<0.05$; [c] $p<0.025$.

A Canadian study evaluated the association between teat variables and the duration of adherence of a dry cow teat seal (Leslie *et al.*, 1999). Each teat of mid-dry period cows and late gestation heifers were randomly assigned to one of 20 experimental groups. Three of these groups involved the use of a commercially available external teat seal based on tetrahydrofuran (as was the one used by Timms *et al.* (1997)), while the others consisted of variations of that commercially available teat seal formulation. Intervals of 24 ± 2 h for14 days were used to assess the adherence of the teat seals. The average number of days the teat-ends were covered was 6.3 ± 0.1 for all formulations. It was found that formulation, teat-end lesion score and teat length significantly influenced the duration of teat seal adherence; but number of days dry, days prior to parturition and teat shape were not significantly associated with teat seal adherence (Leslie *et al.*, 1999).

Another study assessed the efficacy and adherence to the teat of a commercially available teat seal (the same as used by Timms (2001)) in organic herds under natural exposure conditions (Hayton and Bradley, 2001). The teat seal was applied at dry-off to either right or left quarters while the other quarters served as untreated controls. Ten days prior to the estimated date of calving, all teats were dipped in the teat seal and re-dipped as needed until calving, with the same quarter scheme. The persistence of the product was assessed twice weekly. At dry-off, it was shown that the seal persisted for longer on cows giving less than 10 litres/day at dry-off and on front quarters than rear quarters. This difference between front and rear quarters was not observed in the pre-calving period, possibly reflecting the changes in teat placement and udder conformation that occurred in the dry period (Hayton and Bradley, 2001). More than 80% of quarters were protected after three days of drying-off and 21% were still covered one week post-dry-off.

Critical factors in the performance of teat seals are the persistence of the external teat seal on the teat and the resulting protection of the teat orifice. Thus, the development of a formulation that would last for a longer time on the teats would be of great benefit to the dairy industry.

2.5.5. Immunization regimes

Elimination of chronic IMI, prevention of establishment of new IMI and reduction of the frequency and severity of clinical mastitis cases are expected to be the roles of mastitis vaccines (Sordillo *et al.*, 1997). Over recent years, many studies have been directed towards development and improvement of vaccines for mastitis prevention

(Sordillo *et al.*, 1997), with a special focus on the prevention of *E. coli* and *Staph. aureus* (Pyörälä, 2002).

Although mastitis vaccine technologies have improved, these vaccines are not widely used in programs for mastitis control (Sordillo *et al.*, 1997). A core antigen J5 vaccine has been developed to prevent coliform mastitis but its efficacy in reducing mastitis produced by coliforms is still controversial (Sordillo *et al.*, 1997). So far, the development of vaccines against streptococcal species has had little success (Pyörälä, 2002). Attention has been focused on developing vaccines against *Staph. aureus*, but to date, there has been little success (Pyörälä, 2002).

A reason why vaccines may not be effective against mastitis microorganisms may be the high number of mastitis pathogens and their heterogeneity (Pyörälä, 2002).

Research on vaccines to prevent mastitis has been mainly done in the USA, where coliforms are important pathogens causing mastitis in dairy cows. However, coliforms are rarely seen in New Zealand, and vaccination programmes are not implemented in this country.

2.6. Use of cyanoacrylates in animals

Cyanoacrylates were first produced in 1949 but it was not until 1959 that they were proposed for surgical use (Davis *et al.*, 2001). Cyanoacrylates have been extensively used as tissue adhesives (Makady *et al.*, 1991) and as a 'no needle' alternative to conventional suturing in humans (Mattick, 2002). They are liquid monomers that polymerize on contact with tissue surfaces in an exothermic reaction (Singer and

Thode, 2004). They form an insoluble flexible film that adheres strongly to wet surfaces (Makady *et al.*, 1991).

Cyanoacrylates have a common structure, but slight variations in the alkyl group (i.e. methyl, ethyl, butyl, octyl, etc) can change the properties of each individual tissue adhesive (Mattick, 2002). The short-chain cyanoacrylates, such as methyl and ethyl cyanoacrylates, result in histotoxicity; therefore, their clinical use was hindered (Mattick, 2002). It was not until the development of longer-chain cyanoacrylates, first butylcyanoacrylate and then octylcyanoacrylate, that tissue adhesives became useful clinically (Mattick, 2002).

Octylcyanoacrylate is a recent generation that improves on the butylcyanoacrylates (Penoff, 1999). Octylcyanoacrylate is a pliable, waterproof, film adhesive that can be applied to a laceration (Penoff, 1999). In humans, it is appropriate for closure of incisions under casts; for simple lacerations in children to avoid suturing and suture removal; for closure of incisions or lacerations where follow-up is a problem (Penoff, 1999); for any low-tension surgical incision or laceration whose edges are easily approximated with the operator's fingers; for closure of flaps and fragile skin since it does not tear through tissues; for use in place of sutures or staples to secure partial thickness skin grafts; for patients who tend to form hypertrophic scars or keloids (Singer and Thode, 2004).

In animals, octylcyanoacrylate has been evaluated for the treatment of contaminated surgical incisions, burns, abrasions, excisional wounds, split thickness skin grafting, arterial repair and middle ear surgery (Singer and Thode, 2004). However, there is no

published research on the evaluation of octylcyanoacrylate as a method to seal the teat-end in cows.

3. AIM OF THIS STUDY

It is widely recognized that the first weeks after dry-off is the period of highest risk for new intramammary infections. A management strategy to reliably assist dairy farmers with the cessation of milk production at the end of lactation and that would promote efficient mammary gland involution and teat canal closure after drying-off, could result in a significant reduction in the need for blanket antibiotic treatment to prevent new infections in the early dry period. The use of an external teat seal could be a method of achieving these goals to prevent new infections in the early dry period.

Therefore, the objective of this study was to evaluate the effects of an external teat seal based on tetrahydrofuran and a commercially available wound sealant for humans based on 2-octylcyanoacrylate in protecting against mastitis caused by experimental challenge with *Streptococcus uberis* after dry-off, under dairy farming conditions in New Zealand.

4. MATERIALS AND METHODS

4.1. Study design

This study was a prospective, randomized, controlled trial of the effect of two external teat seals on the incidence of new mastitis infections during the dry period, in quarters challenged by exposure to *Streptococcus uberis* after dry-off. One seal used in this study was a variation of a commercially available product based on tetrahydrofuran with an adhesion promoter added (DryFlex™ (DF), DeLaval, Kansas City, MO, USA). The other seal was a commercially available wound sealant intended for use on humans based on 2-octylcyanoacrylate (Band-Aid Liquid Bandage®(BA), Johnson & Johnson, Australia).

The study was conducted between May and October 2005 on a university dairy farm that is operated under normal commercial field conditions, but with a number of modifications described below.

4.2. Selection of herd

The trial was performed at the No. 4 Dairy Farm of Massey University, which is a dual-purpose commercial and research farm located at Massey University. The farm consists of one herd of approximately 480, predominantly Holstein-Friesian, milking cows that calve seasonally between July and October.

Throughout the milking season before the study period, cows were milked twice daily in a DeLaval 56-bale rotary platform milking parlour, with automatic teat cup

removal. Teats were disinfected for the entire season by spraying with a commercial iodophore solution after milking using an automatic teat sprayer.

The cows grazed pasture that was supplemented with hay or silage as required. The average herd milk production during the 10 months immediately preceding the trial was 6,200 litres/day, i.e. approximately 13 litres/cow/day, or 1.1 kg milksolids (MS)/cow/day. The average milk yield at dry-off approximated 7.5 litres/cow/day. The bulk tank somatic cell count recorded immediately prior to dry-off was 260,000 cells/ml and the average herd BTSCC during the 2004 - 2005 season was 300,000 cells/ml.

4.3. Selection of cows

On the basis of herd testing conducted in April 2005, cows with a somatic cell count of less than 200,000 SCC/ml were identified. From this group, the 180 cows with the lowest SCC and with four functional quarters were deemed eligible for the study, provided they were free of clinical signs of mastitis or teat abnormalities at the time of enrolment. Table 4.1 provides details of the cows enrolled in the study (n = 175), stratified by lactation number.

4.4. Pre-treatment milk sampling

Enrolled cows were identified by ear tag when they entered the milking platform. At four days and one day before the date of dry-off (referred to as day 0 in the remainder of this description), single-quarter milk samples were collected aseptically

Table 4.1. Number of cows in each group, stratified by lactation number, with the number of quarters treated with each product shown in brackets

Lactation	DryFlex (DF)	Band-Aid (BA)	Total
2	26 (52)	25 (50)	51 (102)
3	19 (38)	19 (38)	38 (76)
4	17 (34)	17 (34)	34 (68)
5	9 (18)	9 (18)	18 (36)
6	7 (14)	8 (16)	15 (30)
7	4 (8)	4 (8)	8 (16)
8	2 (4)	2 (4)	4 (8)
9	1 (2)	1 (2)	2 (4)
10	2 (4)	2 (4)	4 (8)
11	1 (2)	0 (0)	1 (2)
Total	88 (176)	87 (174)	175 (350)

by trained personnel before the morning milking (refer to Figure 4.1 for a full description of the timeline of the trial). Disposable latex gloves were worn throughout the milk sampling procedure. Before sampling, teats were wiped with 70% ethanol-dampened-cotton swabs starting with the distant teats. Teat-ends were cleaned until there was no dirt visible on the teat-end and no dirt appeared on the swab. Care was taken to avoid contact with tails, feet and legs once the teats were clean. The teats were allowed to dry for several seconds before sampling. To minimise cross-contamination, milk samples were first taken from the nearest teats, then from the distant teats. The first two or three squirts of milk were discarded and samples were collected in sterile plastic containers with watertight lids. The samples were transported on ice to the microbiology laboratory of the Institute of Veterinary, Animal and Biomedical Sciences (IVABS) of Massey University and were analysed bacteriologically on the same day as sampling.

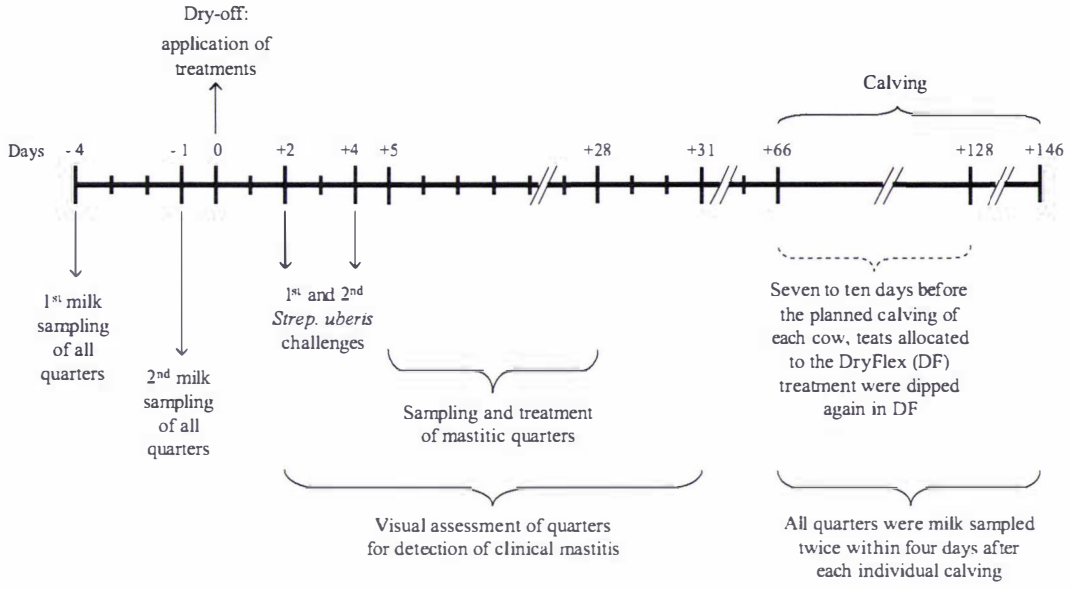

Figure 4.1. Diagram showing dates and treatments of the experiment

4.5. Treatments

All cows were abruptly dried off at the end of the designated morning milking of day 0. Those cows not enrolled in the study received antibiotic dry-cow therapy and were kept in separate paddocks from the enrolled cows.

A systematic stratified random design was used to assign cows to treatment groups. Cows were sorted by lactation number. Within each lactation group, cows were listed in ascending ear tag order and the first cow in each lactation group was assigned to either DryFlex treatment, or Band-Aid treatment by tossing a coin. Subsequent cows were then assigned to treatments in an alternating fashion. All the cows belonging to the same treatment group were pooled, ordered by ascending ear tag number and the contralateral quarters left front/right back (LF-RB) and right front/left back (RF-LB) of the first cow in each treatment group were assigned to one of the two treatments or

to the untreated control (see Figure 4.2) by tossing a coin. Cows were then alternately assigned to the contralateral teat pairs' scheme.

Prior to application of treatments and starting with teats on the part of the udder most distant from the operator, teats were wiped with 70% ethanol-dampened-cotton swabs. Teat-ends were cleaned until no dirt was visible on the teat-end or appeared on the swab. Teats were allowed to dry completely prior to applying the teat seals. To minimise contamination of teats that had not been treated, teat seals were applied to the nearest teats first.

Figure 4.2. Image of an udder showing the contralateral scheme used for the application of the treatments. Left front and right back quarters received the DryFlex treatment, while the right front and left back quarters were left untreated

Teats of cows that received DF treatment were dipped in the teat seal in an applicator cup so that the entire length of the teat was covered, as per the manufacturer's instructions. Any unused product that remained in the applicator cup was discarded. Cups were replaced after use on three cows.

Teats of cows that received the BA treatment were manually inverted to visualize the teat orifice. Four drops of the BA product were applied onto the applicator provided with the product and the applicator was used to wipe the teat to cover a circular surface of about 2 cm diameter on and around the teat orifice. Each applicator was used for a single teat and was discarded after use.

Treatment substances were allowed to dry completely for approximately five minutes before releasing the cows to the paddock. No antibiotic dry-cow therapy was infused into any quarter and teats were not disinfected after the treatments had been applied. Disposable latex gloves were worn throughout the treatment procedure.

4.6. Bacterial challenge

Cows were subjected to two experimental exposures to *Streptococcus uberis* carried out at two and four days after dry-off (see Figure 4.1). The strain used for the challenge was selected haphazardly, i.e. without a formalised random scheme, from a collection of *Strep. uberis* strains previously isolated in New Zealand. The isolate was from a bovine milk sample taken on 12th May 1995 in Horowhenua district, Wellington region, isolated as part of a previous study (Douglas *et al.*, 2000) and kept frozen at -80°C at the IVABS microbiology laboratory. Before the trial, this challenge isolate was cultured on a 5% sheep blood agar plate, checked for purity and re-identified phenotypically using an API 20 STREP kit (bioMérieux® sa, Marcy-l'Etoile, France), which resulted in a 99.9% *Streptococcus uberis* identification.

Four days before each challenge, an aliquot of the frozen challenge strain was thawed in sterile normal saline to form a heavy bacterial suspension that was streaked on 5% sheep blood agar plates using cotton swabs to cover the whole surface of the plate. The plates were incubated at 37°C for 48 h under normal aerobic conditions; bacteria were then harvested using cotton swabs and suspended in 60 ml of normal saline. Six ml of this suspension was aseptically inoculated into each one of ten, 150 ml rectangular canted cell-culture flasks with vent caps (Corning Costar Corporation, Cambridge, MA, USA), containing 20 ml of 5% blood agar medium. The flasks were incubated at 37°C under aerobic conditions for approximately 2 h with the blood agar medium on the underside to avoid its detachment from the flask. Finally, the excess fluids were discarded and the flasks re-incubated with the blood agar medium on the underside for 48 h as before.

Strep. uberis bacteria were harvested on the day of each challenge, as follows: five to ten ml of sterile saline and sterile spherical glass beads were aseptically transferred into the flasks. Flasks were gently agitated to detach the adhering bacterial slime from the surface of the agar and the resulting bacterial suspension was poured into a sterile bottle.

In the bottle, the suspension was adjusted to a final volume of approximately 1.5 litres using sterile normal saline. A pilot experiment conducted prior to the trial resulted in a bacterial suspension of turbidity greater than that of the 0.5 McFarland turbidity standard, suggesting a bacterial density greater than $1x10^{7}$ colony forming units per ml (cfu/ml). This volume corresponded to the calculated volume of bacterial suspension needed to expose, by dipping, all the cows. Approximately 60

ml of this bacterial suspension was transferred into each one of a series of clean plastic containers with watertight lids, which were then transferred on ice to the farm on the same morning. A small aliquot of the final bacterial suspension was subjected to bacterial cfu counting, using the serial dilution method. One ml of the bacterial suspension was diluted tenfold in normal saline, resulting in a suspension of a turbidity corresponding to approximately the 0.5 McFarland turbidity standard. Tenfold serial dilutions were performed and 100 μl of the 10^{-5}, the 10^{-6} and the 10^{-7} dilutions were spread, in triplicate, on 5% blood agar plates that were incubated at 37°C for 48 h under aerobic conditions. The arithmetic average of the number of the colonies that grew on the dilution showing between 30 - 300 cfu on the plates was used to calculate the bacterial density expressed in cfu/ml used for the experimental exposure of cows by dipping. The results indicated a first experimental exposure with 1.27×10^8 *Strep. uberis* cfu/ml and second exposure with 1.02×10^8 cfu/ml.

For the experimental challenge, the enrolled cows were taken to an old milking parlour, identified by ear tag and checked for the presence of clinical mastitis as described in the next section. Then, each of the four teats, starting with the front ones, was dipped in bacterial suspension for 1-2 seconds and the cow was released. Containers were changed every 5 to 7 cows, as the fluid levels in the bottles dropped. Care was taken to discard the unused bacterial suspension into a disinfectant solution to avoid excessive contamination of the facilities with the challenge bacterium. These procedures were used on both days of challenge.

4.7. Post-challenge clinical assessments

After the first experimental challenge, the persistence of the teat seal used for DF treatment was monitored by a daily visual inspection of the teats, until no treatment was visible on any teat. Persistence of the seal was assessed using a five-point visual adherence rating previously reported by Hemling *et al.* (2005) on the manufacturer's internet site (www.westagro.com accessed on 5th April 2005). According to the scheme, a score of 5 corresponds to a tightly covered teat with very little or no signs of wear in the seal; values 4 to 2 refer to increasing degrees of rolling or tearing of the teat seal and 1 represents a teat that has completely lost the teat seal (Figure 4.3). According to the manufacturer, values between 2 to 5 provide equal and complete protection to the teat orifice.

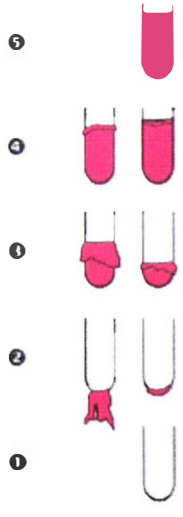

Figure 4.3. Diagram representing the five-point visual assessment score for teat seal adherence reported by Hemling *et al.* (2005)

The product used for BA treatment was translucent and had a texture similar to that of skin, so it was not visually obvious. Thus, its presence/absence was unable to be recorded satisfactorily.

Examinations for detection of clinical mastitis were performed on all quarters every day by an experienced dairy technician, starting on the day of the first bacterial challenge and continuing until the last observation on day 31 (see Figure 4.1). Individual quarters were observed and palpated for the presence of clinical signs consistent with mastitis, i.e., heat, swelling, a painful udder and if required, by an examination for the presence of clots and flakes in the secretions. Each quarter was subjectively judged as having clinical mastitis or not, according to these criteria. The technician in charge of assessing clinical mastitis was unaware of the treatment each quarter had received. However, due to the nature of the treatments, it is possible that this person did become aware of the treatment that some quarters had received since they were visibly obvious. This fact was taken into account in the analysis reported below.

Mastitic quarters were sampled for bacteriological culture as described in section 4.4. The quarters were stripped out completely and treated according to veterinary protocols as follows: a) if only one quarter was inflamed, it was treated with daily intramammary infusions containing oxytetracycline hydrochloride, oleandomycin, neomycin and prednisolone (Mastalone, Pfizer Laboratories Ltd, NZ), for three days; b) if more than one quarter was inflamed, cows were treated with daily intramuscular injections of penethamate hydriodide (Mamyzin, Boehringer Ingelheim Ltd, NZ) for three consecutive days.

Treatment (a) was interrupted and treatment (b) initiated if one or more additional quarters were declared mastitic, in a cow previously affected with mastitis in a single quarter. Teats were sprayed with iodine after each mastitis treatment.

4.8. Pre-calving treatment

Seven to ten days before calving, teats allocated to the DF treatment were dipped in DF again, according to the manufacturer's instructions using the same contralateral scheme as for the dry-off treatments (see Figure 4.1). To avoid unnecessary stress in peri-parturient cows, the persistence and appearance of the DF treatment were not monitored after the repeat treatment.

4.9. Post-calving milk sampling

Single-quarter milk samples were taken aseptically on two occasions, within four days after calving (see Figure 4.1), as described in section 4.4.

4.10. Bacteriological analysis

4.10.1. Bacterial isolation

Milk samples were processed according to the National Mastitis Council guidelines (Anon., 1999). Ten microlitres of milk were deposited on one quadrant of a 5% sheep blood agar bacterial culture plate (Fort Richard Laboratories Ltd, Auckland, NZ) by means of sterile tips and a micropipette. The milk was streaked to cover the quadrant's surface using a sterile glass spreader. Samples from the four quarters of each cow were inoculated onto four quadrants of the same plate. The plates were incubated at 35-37°C under aerobic conditions for 48 h. After incubation, the plates were inspected and results recorded according to the following criteria:

a) Growth of more than two colony types on the quadrant: sample considered contaminated and no further actions taken.

b) A maximum of two colonies growing on the quadrant: an uninfected sample; no further actions taken.

c) More than two similar colonies (but less than three colony types) growing on the quadrant: an infected sample; one colony of the predominant type was isolated on a new blood agar plate.

Colony types requiring isolation were picked and subcultured on the entire surface of a new 5% sheep blood agar plate. The isolates were grown for 48 h, as before. Then heavy bacterial suspensions were prepared in 15% glycerol broth and frozen in cryogenic vials at -80°C for future reference.

4.10.2. Bacterial identification

Approximately two months after the conclusion of the trial, the frozen isolates recovered from the post dry-off and post-calving milk samples were subjected to an identification scheme using a series of conventional biochemical reactions. Isolates recovered from the pre dry-off samples were not subjected to bacterial identification.

The bacterial identification scheme aimed to differentiate between *Strep. uberis* and other mastitis-causing bacteria. It involved a culture of the previously frozen isolates on 5% blood aesculin plates incubated for 24 h at 35-37°C in aerobic conditions, followed by a Gram stain and the testing for the catalase reaction. All Gram-positive, catalase-positive cocci were subjected to a standard tube rabbit plasma coagulase test, with overnight incubation at 35-37°C under aerobic conditions. All Gram-positive, catalase-negative and aesculin-positive cocci were subjected to an inulin fermentation test and a buffered azide glucose glycerol (BAGG) test with incubation of 24 h at 37°C under aerobic conditions (Hajna, 1951). Inulin positive and BAGG negative bacteria were identified as *Strep. uberis*. Gram-positive, catalase-negative

and aesculin-negative cocci were subjected to a CAMP test, to identify possible *Strep. agalactiae* isolates (Quinn *et al.*, 1994). Other Gram-positive, catalase-negative bacteria were not further identified. Bacteria that were not Gram-positive cocci were only defined by their cell morphology and Gram stain reaction. *Corynebacterium bovis* was identified based on its typical colony characteristics and Gram-stain reaction. They were identified as opaque and greyish, non-haemolytic colonies of a chalky consistency (Anon., 1999). One isolate had its identification confirmed as *C. bovis* by means of an API Coryne test kit (bioMérieux® sa, Marcy-l'Etoile, France).

4.11. Additional data collection

The following data were recorded for each cow enrolled in the trial: parity, last recorded daily milk production, last SCC prior to drying-off, drying-off date, calving date. The date of withdrawal and the reason for withdrawal from the trial, of any cow were also recorded.

4.12. Animal ethics

This study was approved by the Massey University Animal Ethics Committee (Protocol 05/40).

4.13. Pulsed-field gel electrophoresis of *Streptococcus uberis* chromosomal DNA macro-restriction fragments

The production of restriction endonuclease fragment patterns using pulsed-field gel electrophoresis (PFGE) is a useful tool for subtyping *Strep. uberis* isolates (Douglas *et al.,* 2000). Therefore, in order to assess whether the quarters showing clinical

mastitis were infected with the *Strep. uberis* organism used for the experimental exposure, the chromosomal restriction endonuclease banding patterns of selected *Strep. uberis* isolates were determined using PFGE, according to McEllistrem *et al.* (2000) and Smith and Cantor (1987), with some modifications.

Nineteen frozen isolates from post dry-off milk samples collected from quarters showing clinical mastitis were haphazardly selected and re-identified as *Strep. uberis* by a battery of phenotypic tests, which included an aesculin hydrolysis test, a Gram stain reaction, a catalase test, an inulin fermentation test and a BAGG test. These isolates, the infection strain and an epidemiologically-unrelated *Strep. uberis* control strain taken from the same culture collection as the challenge strain, were analysed by PFGE.

In the description of the results of PFGE that follows, the terms "indistinguishable" (meaning they have the same banding pattern), "closely related" (showing up to 3-bands difference), "possibly related" (showing a 4- to 6-band difference) and "unrelated" (when there are more than six bands difference) are used in comparing bacterial isolates taken from post-dry-off milk samples. This designation is as for Tenover *et al.* (1995).

4.13.1. DNA plug preparation

The 19 field isolates, together with the challenge and control organisms, were allowed to grow for 48 h on 5% blood agar plates incubated at 35-37°C. Bacteria were harvested by means of cotton swabs and placed into 3 ml vials of brain heart infusion (BHI) broth. The optical density (OD) of the bacterial suspension was

measured at 610 nm using a spectrophotometer (Unicam Limited, Cambridge, UK) and adjusted to an OD of 1.4. A 100 µl aliquot of the bacterial suspension was placed in an eppendorf tube and centrifuged at 13,000 rpm (12,000 g) for 5 minutes. The supernatant was carefully removed and the precipitate was resuspended in 150 µl of cold cell suspension buffer (1 M NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]) and centrifuged again at 13,000 rpm for 5 minutes. The supernatant was removed and the precipitate was re-suspended in 50 µl of cold cell suspension buffer. The suspension was mixed with 100 µl of 2% melted low-melt agarose (Bio-Rad Laboratories, Hercules, CA, USA). One hundred microlitres of this mixture were carefully placed into the wells of reusable plug moulds (Bio-Rad Laboratories) and were then cooled on ice for 1 h. Once solidified, plugs were placed in eppendorf tubes with 1 ml of lysis buffer 1 (1M NaCl, 100 mM EDTA [pH 8.0], 6 mM Tris-HCl [pH 8.0], 0.5% Brij 58, 0.5% sodium deoxycholate, 0.5% sodium lauroyl sarcosine) containing 1 mg/µl of lysozyme (Roche Diagnostics, Indianapolis, IN, USA) per ml and incubated in a water bath (Bellco Biotechnology, Vineland, NJ, USA) at 37°C, overnight. Following incubation, lysis buffer 1 was carefully removed and 1 ml of ESP buffer (0.5M EDTA, 1% sodium lauroyl sarcosine) containing 100 µg of proteinase K (Roche Diagnostics) per ml was placed into each tube and incubated overnight in a water bath at 56°C. The plugs were then washed 4 times with 10 ml of TE buffer (10 mM Tris-HCl, 1mM EDTA) for 1 h each time on ice on a rocking machine (Bellco Biotechnology) and stored in eppendorf tubes containing 1 ml of TE buffer at 4°C overnight.

4.13.2. DNA digestion

Each plug was removed from the tube and placed on a sterile glass slide. A 2 mm-wide slice, corresponding to about one third of the plug, was cut with a scalpel and placed into a new eppendorf tube containing 100 µl of cutting buffer (10 µl restriction buffer 4 [10x New England Biolabs, Beverly, MA, USA], 30 Units SmaI [New England Biolabs], 88.5 µl sterile MQ water) and equilibrated on ice for 45 minutes. Ethanol was used to disinfect and clean both the glass slide and scalpel after slicing each plug. These preparations were incubated at 25°C for 24 h. The remaining two-thirds of each plug was placed into 1 ml of TE buffer in eppendorf tubes and stored at 4°C for future reference.

4.13.3. PFGE conditions and gel analysis

The electrophoresis cell of a contour-clamped homogenous electric field (CHEF Mapper machine) (Bio-Rad Laboratories) was filled with 2.2 litres of 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.0]) and circulated for about 1 h at 12°C. The restriction digested plugs were loaded in a 1% pulsed field certified agarose gel (Bio-Rad Laboratories) along with 2 lambda ladder PFG markers (New England Biolabs) and one low-range PFG marker (New England Biolabs), which were included as molecular size standards. The macro-restriction fragments were separated by PFGE with a pulse angle of 120°, a gradient of 6V/cm and a 23-h run. Initial and final switch times of 1 and 40 s, respectively and a buffer temperature of 12°C were used.

On completion, the gel was immersed in ethidium bromide solution for 10 minutes, then briefly rinsed in sterile distilled water and photographed under UV illumination

using the Gel Doc 2000 (Bio-Rad Laboratories). The bands were defined using the software Diversity Database (Bio-Rad Laboratories, USA), with a maximum position tolerance of 1%. Bands were described by their sizes in kilobases (kb) relative to the infection strain and were identified from the higher molecular weight bands to the lowest. Band positions in the digitised gel image were determined automatically by computer but were then confirmed visually and corrected manually. The DNA banding patterns of the tested strains were visually compared with the pattern of the infection strain.

4.14. Statistical analyses

Considering each quarter an independent unit (Woolford *et al.*, 1998), the incidence of clinical mastitis in the treated and untreated quarters was first compared using relative risks (RR) and 95% confidence intervals (CI) and the probabilities of such proportional differences were calculated using Rothman's Episheet (Rothman, 2002). Relative risks were calculated on the raw data. In addition, in order to control for possible misclassification bias due to the obvious presence of the product DF on some teats, RR were also calculated using only cows in which mastitis was not diagnosed, or was only diagnosed after the product was no longer visible on the teats. Survival analyses were used to evaluate the effect of treatment on clinical mastitis hazard throughout the follow-up period. The event of interest was the date of first detection of clinical mastitis in a quarter. Quarters were right-censored at the end of the follow up period if clinical mastitis was not observed. The effect of parity (first lactation cows vs. other cows), pre-dry off bacteriological status (infected vs. not infected) and treatment status (treatment vs. control) on the number of days from the start of the follow up period to detection of clinical mastitis was assessed using

Kaplan-Meier survival curves and the Log Rank statistic. Explanatory variables associated with the event of interest at $P < 0.20$ were selected for inclusion in a Cox proportional hazards regression model. Regression coefficients were determined using a forward-stepwise approach using the coxph procedure in R version 2.1.0 (R Development Core Team, 2005). The significance of the addition of explanatory variables into the model was tested using a likelihood-ratio test. Explanatory variables that were significant at $P < 0.05$ remained in the model. The assumption of proportionality of hazards was assessed using the Pearson product-moment correlation between the scaled Schoenfeld residuals for each explanatory variable and time. To account for the hierarchical structure of the data (that is, quarters clustered within cows) and the possibility of correlation of time to event in individuals the fixed-effects Cox model was extended to include a Gaussian distributed frailty term for cow (Therneau and Grambsch, 2001).

5. RESULTS

5.1. Clinical assessment of mastitis

5.1.1. Pre-dry-off clinical assessment

Clinical mastitis was not seen in any quarter of the 175 cows during the pre-dry-off sampling.

5.1.2. Post-dry-off clinical assessment

All 175 cows enrolled in the study were assessed for clinical mastitis from day 2 post-dry-off. Data on mastitis status post-dry-off are available for 700 quarters, of which 352 are from the DryFlex (DF) group and 348 are from the Band-Aid (BA) group. In total, clinical mastitis was detected in 249 quarters (35.6%) in 136 (77.7%) cows during the entire observation period. Following bacterial challenge with *Strep. uberis*, in the DF group, clinical mastitis developed in 35 of the treated quarters and 83 of the untreated quarters (Table 5.1). The corresponding data for the BA group are 67 of the treated quarters and 64 of the untreated quarters. Clinical signs of mastitis were first seen on days 5 and 6 after dry-off for the DF and BA groups, respectively (Table 5.1). The last case of clinical mastitis for the DF and BA groups was recorded on day 26 and day 28, respectively. Figure 5.1 shows that the incidence of mastitis was greatest between days 6 and 11 after dry-off for both groups. This figure presents data for the first 17 days after dry-off because most of the clinical mastitis cases were found within those days.

Table 5.1. Number of quarters detected with clinical mastitis by treatments on each day after dry-off

Days after dry-off	Treatment				Total
	DryFlex		Band-Aid		
	Treated	Control	Treated	Control	
1	0	0	0	0	0
2	0	0	0	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	1	0	0	1
6	1	3	7	4	15
7	5	18	14	10	47
8	7	18	28	15	68
9	10	19	9	21	59
10	6	9	6	5	26
11	2	4	1	3	10
12	2	0	0	1	3
13	1	4	1	1	7
14	0	0	0	1	1
15	1	1	0	1	3
16	0	1	0	0	1
17	0	2	0	0	2
18	0	0	0	0	0
19	0	0	0	0	0
20	0	1	0	0	1
21	0	0	0	0	0
22	0	1	0	0	1
23	0	0	0	2	2
24	0	0	0	0	0
25	0	0	0	0	0
26	0	1	0	0	1
27	0	0	0	0	0
28	0	0	1	0	1
Total	**35**	**83**	**67**	**64**	**249**

Considering a quarter as an individual observation, the risk of mastitis in the DF group was higher in control quarters than in the quarters treated with DF (RR: 2.37, 95% CI 1.70 - 3.32, $P = 0.000$). When only those cows in the DF group in which

mastitis was diagnosed when the product was no longer visible on any of the teats are considered, the RR of mastitis was still higher in control quarters than in the quarters treated with DF (RR: 1.83, 95% CI 1.24 - 2.68, p = 0.0015). The difference between these RRs in the DF group is probably due to the protection conferred by the product against early intramammary infections, but we cannot rule out a certain amount of misclassification bias in those cows in which mastitis was diagnosed when the product was still visible.

There was no significant difference between the treated and untreated quarters in the BA group in the number of new clinical mastitis acquired during the dry period (RR: 0.96, 95% CI: 0.73, 1.25, $P = 0.74$).

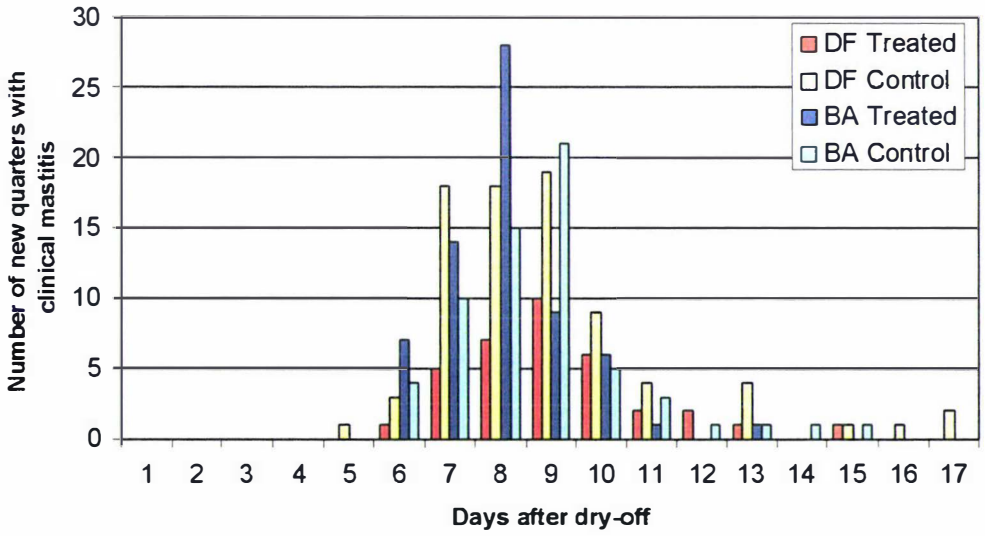

Figure 5.1. Number of new quarters found with clinical mastitis after dry-off. DF: DryFlex; BA: Band-Aid

Kaplan-Meier survival curves showing the cumulative proportion of quarters that remained mastitis-free as a function of the number of days from dry-off for the DF and BA groups are shown in Figures 5.2 and 5.3, respectively. In the DF group, 35 events of clinical mastitis occurred in 176 treated quarters, compared with 83 events among the 176 untreated quarters. The difference in time to the observation of clinical mastitis between treatment and control quarters was significant (log rank test statistic 30.2; df 1; $P < 0.01$).

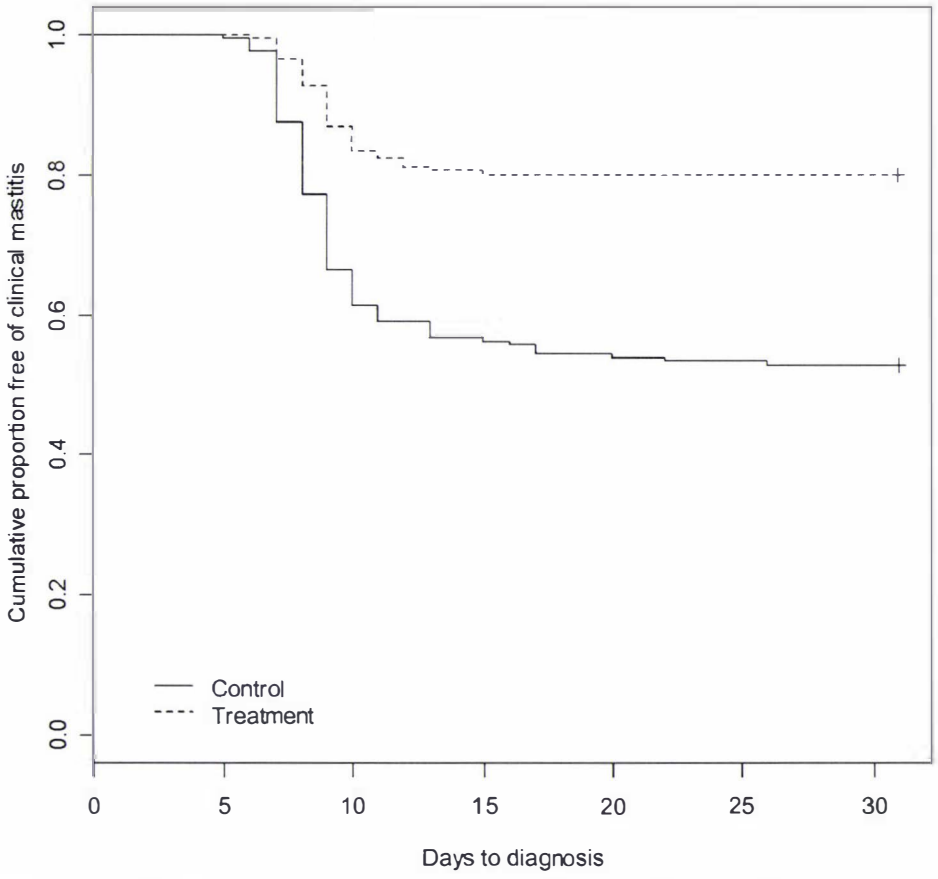

Figure 5.2. Kaplan-Meier survival curves showing the cumulative proportion of quarters that remained mastitis-free as a function of the number of days from treatment for DryFlex treated quarters

To remove the effect of any differential tendency to diagnose mastitis events in untreated quarters on account of the visible evidence of DryFlex on treated quarters

remaining for up to 10 days, analyses were re-run after shifting the start of the observation period to dry-off date plus 10 days. For this data set, the difference in time to the observation of clinical mastitis between treatment and control quarters was significant (log rank test statistic 7.7; df 1; $P < 0.01$).

For the BA group, 67 clinical mastitis events were recorded in 174 treated quarters compared with 64 clinical mastitis events in 174 untreated quarters. The difference in time to clinical mastitis event between treatment and control quarters was not significant at the alpha level of 0.05 (log rank test statistic 0.40; df 1; $P = 0.52$).

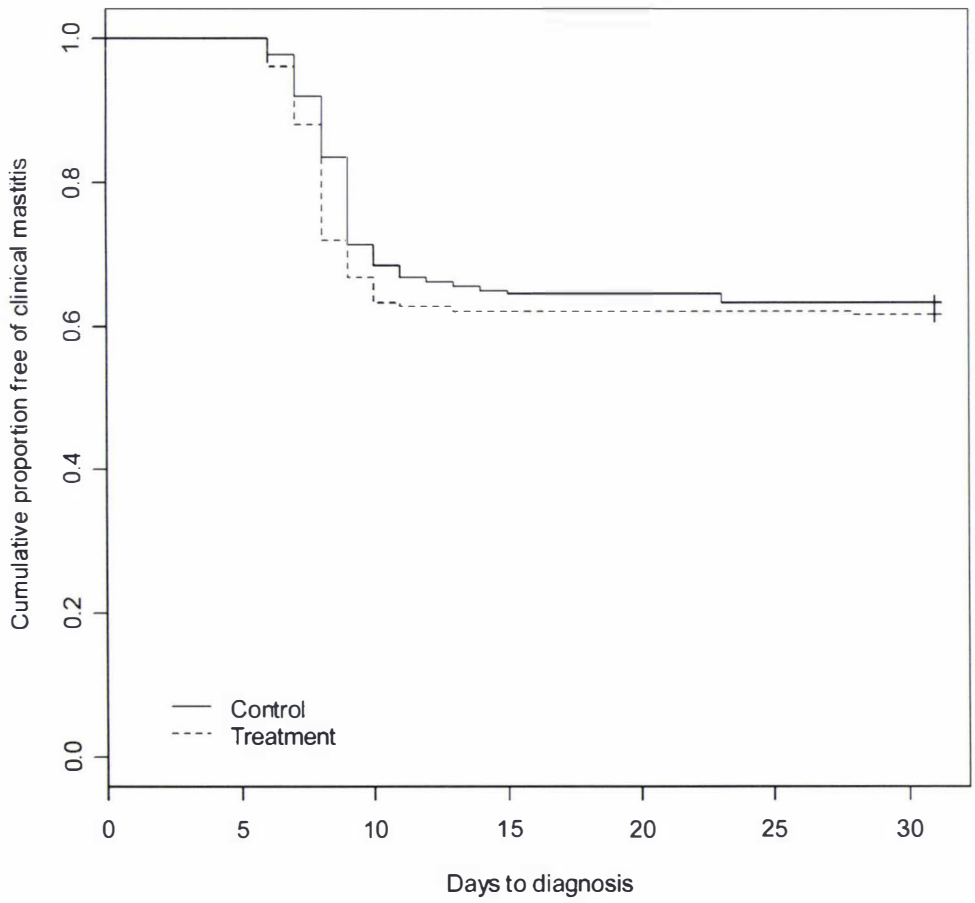

Figure 5.3. Kaplan-Meier survival curves showing the cumulative proportion of quarters that remained mastitis-free as a function of the number of days from treatment for Band-Aid treated quarters

For the DF group, the difference in time to clinical mastitis for treated and control quarters differed according to their pre-dry-off bacteriological status (log rank test statistic 7.4; df 1; $P < 0.01$) (see Table 5.2). Seventy five of the 187 quarters that were classified as infected, contaminated, or not sampled at days four or one before dry-off experienced mastitis during the follow-up period, compared with 43 of 165 quarters classified as not infected. For the DF group, time to observation of clinical mastitis for treated and control quarters did not differ between primiparous and multiparous cows (log rank test statistic 0.90; df 1; $P = 0.34$).

Table 5.2 presents the results of the Cox proportional hazards regression, quantifying the effects of treatment and pre-dry-off bacteriological status on the hazard of experiencing clinical mastitis for the DF group during the observation period. After adjusting for the effect of bacteriological status pre-dry-off and individual cow effects, the daily hazard of mastitis in control quarters was 2.97 (95% CI 2.00 - 4.42) times that of quarters treated with DryFlex. After adjusting for the effect of treatment status and individual cow effects, the daily hazard of mastitis in quarters identified as infected before dry-off was 1.64 (95% CI 1.10 - 2.44) times that of uninfected quarters.

For the BA group, the difference in the time to observation of clinical mastitis for treated and control quarters differed according to the pre-dry-off bacteriological status (log rank test statistic 22.5; df 1; $P < 0.01$). Eighty two of the 164 quarters that were classified as infected, contaminated, or not sampled at days four or one before dry-off experienced mastitis during the follow-up period, compared with 49 of 184 quarters classified as not infected. For the BA group, the difference in time to clinical

mastitis event for treated and control quarters differed between primiparous and multiparous cows (log rank test statistic 7.4; df 1; P = 0.006). Twenty six of the 100 quarters from primiparous cows experienced mastitis during the follow-up period, compared with 105 of 248 quarters from multiparous cows.

Table 5.2. For the DryFlex group: Cox proportional hazards model showing the effect of treatment (DryFlex treated or control) and bacteriological status before dry-off on the daily hazard of experiencing clinical mastitis

Variable	Subjects	Infected	Coefficient (SE)	P	Hazard ratio (95% CI)
Group					
Control	176	83	1.0890 (0.2020)	< 0.01	2.97 (2.00 – 4.42)
Treatment	176	35	-		1.00
Bacteriological status before dry-off					
Positive	187	75	0.4940 (0.2040)	0.01	1.64 (1.10 – 2.44)
Negative	165	43	-		1.00

$R^2 = 0.205$
Variance of random effect: 0.217
Likelihood ratio test = 80.9 on 21.3 df, P < 0.01

Table 5.3 presents the results of the Cox proportional hazards regression model quantifying the effect of treatment, parity and pre-dry-off bacteriological status on the hazard of experiencing clinical mastitis for the BA group during the observation period. After adjusting for the effect of pre-dry-off bacteriological status and parity, the daily hazard of mastitis in control quarters was 1.13 (95% CI 0.62 – 1.24) times that of quarters treated with Band-Aid. After adjusting for the effect of treatment status and parity, the daily hazard of mastitis in quarters identified as infected pre-dry-off was 2.15 (95% CI 1.50 – 3.09) times that of uninfected quarters. After adjusting for the effect of treatment and pre-dry-off bacteriological status, quarters

from multiparous cows had 1.50 (95% CI 0.97 – 2.33) times the daily hazard of mastitis, as quarters from primiparous cows.

Table 5.3. For the Band-Aid group: Cox proportional hazards model showing the effect of treatment (Band-Aid or control), lactation number and bacteriological status of milk samples pre-dry-off on the daily hazard of experiencing clinical mastitis

Variable	Subjects	Infected	Coefficient (SE)	P	Hazard ratio (95% CI)
Group					
Control	174	64	-0.1270 (0.1750)	0.47	1.13 (0.62 – 1.24)
Treatment	174	67	-		1.00
Lactation number					
Primipara	100	26	-		1.00
Multipara	248	105	0.4070 (0.2240)	0.07	1.50 (0.97 – 2.33)
Bacteriological status before dry-off					
Positive	164	82	0.7680 (0.1850)	0.01	2.15 (1.50 – 3.09)
Negative	184	49	-		1.00

Figure 5.4 shows the point estimate and 95% confidence interval of the hazard estimates of the 88 cow-level effects included in the DF group, plotted in order of rank (lowest to highest). Figure 5.4 shows that the daily hazard of mastitis attributable to individual-level effects varied from 0.71 (95% CI 0.32 - 1.57) to 1.71 (95% CI 0.72 - 4.07). The overlapping confidence intervals shown in Figure 5.4 indicate that although individual-level effects varied among the animals included in the study, they were not significantly different at the alpha level of 0.05.

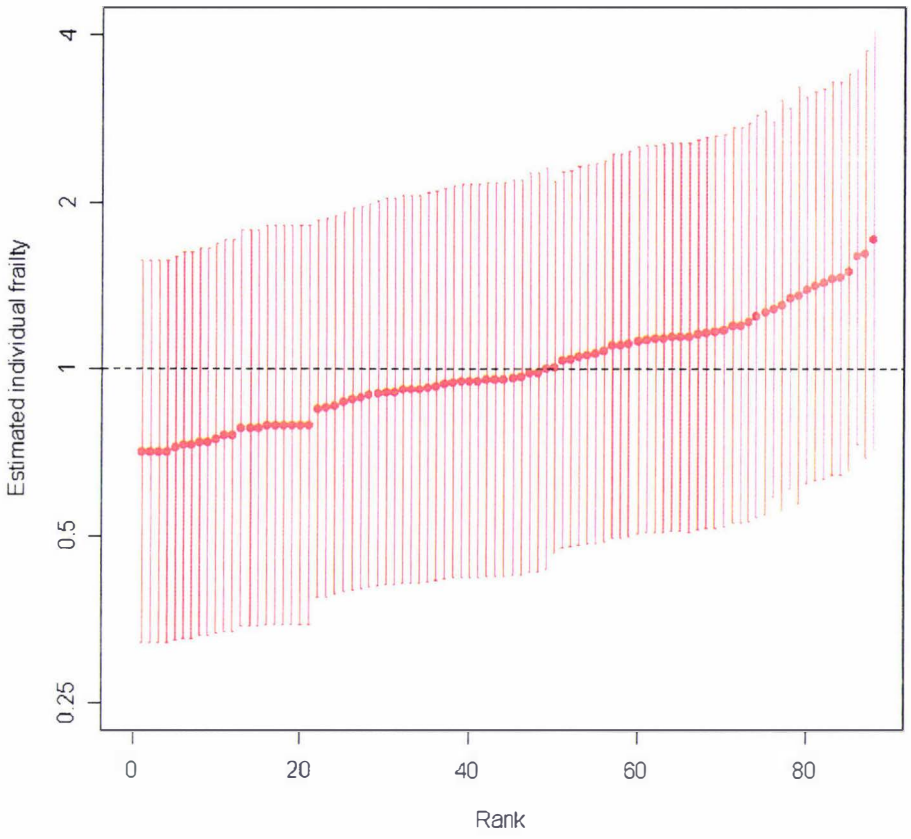

Figure 5.4. Error bar plot showing the point estimate and 95% confidence intervals of the hazard estimated for the 88 cows included in the DryFlex group, in order of rank (lowest hazard to highest hazard, left to right)

5.1.3. Post-calving clinical assessment

In the DF group only two of the six quarters (three treated with DF and three untreated) from five cows that showed signs of clinical mastitis after calving, had previously shown clinical mastitis after dry-off and these dry period infections were due to *Strep. uberis* (one of them had mastitis post-calving due to *Strep. uberis*). The other four quarters did not previously have clinical mastitis after dry-off.

In the BA group, ten quarters (five treated with BA and five untreated) from nine cows showed signs of clinical mastitis post-calving. Of these, five quarters had previously had clinical mastitis post-dry-off when three of them were due to *Strep. uberis*. The other five quarters had not previously had mastitis post-dry-off.

5.2. Bacteriological results

5.2.1. Pre-dry-off bacteriological results

Pre-dry-off bacteriological results by treatment are reported in Table 5.4.

Table 5.4. Number of quarters by infection status and treatment at four and one day(s) before the application of DryFlex or Band-Aid treatments at drying-off

Infection status	Treatment			
	DryFlex		Band-Aid	
	4 days before dry-off	1 day before dry-off	4 days before dry-off	1 day before dry-off
Non-infected	118	109	132	131
Infected	22	33	27	36
Corynebacterium bovis	127	137	108	121
Contaminated	65	69	77	60
Missing	20	4	4	0
Total	352	352	348	348

On days -4 and -1, there were 28 missing quarter samples from seven cows that were not available because they were not present on the farm for milk samplings, due to logistical circumstances that were unrelated to the trial.

Intramammary infections were diagnosed on both days in seven quarters of seven cows in the DF group and in ten quarters of eight cows in the BA group.

Interestingly, *Corynebacterium bovis* was highly prevalent in the herd on both sampling days (Table 5.4). *C. bovis* is generally considered a colonizer of the teat canal, which only rarely causes clinical mastitis (Anon., 1999).

5.2.2. Post-dry-off bacteriological results

Bacteriological analysis was conducted on 169 single-quarter milk samples. Due to samples misplaced in the laboratory, 80 milk samples could not be analysed, 64 of which were taken on day 8. *Strep. uberis* was the most prevalent bacterium on days 6-7 and from day 9 onwards, and it was detected in 160 (95%) single-quarter milk samples. No coagulase-positive staphylococci were found after dry-off.

Table 5.5 shows the bacteriological results of milk samples taken from mastitic quarters during the post-dry-off observation period for both experimental groups.

Table 5.5. Description of bacterial species isolated from quarters with clinical mastitis after dry-off by treatment

	Treatment			
	DryFlex		Band-Aid	
	Treated	Control	Treated	Control
Streptococcus uberis	21	59	37	43
Other Gram (+)ve, catalase (-)ve cocci	0	0	0	2
Coagulase-negative staphylococci	0	1	1	2
Gram-positive bacilli	2	0	0	0
Gram-negative bacilli	1	0	0	0
Missing	11	23	29	17
Total	35	83	67	64

5.2.3. Post-calving bacteriological results

In the DF group, four of the 175 cows enrolled in the study were not sampled post-calving. Two died soon after calving for causes unrelated to the study and two aborted and were eliminated from the herd. Similarly, in the BA group, five cows were not sampled post-calving. One died soon after calving for causes unrelated to the study, three were declared as non-pregnant at the end of the trial and one aborted and was eliminated from the herd.

The bacteriological results of the post-calving milk samples from both experimental groups are summarised in Table 5.6. *Strep. uberis* was the most prevalent species found in post-calving milk samples. *Streptococcus agalactiae* was not found. There were a few coagulase-negative and coagulase-positive staphylococci, as well as Gram-positive and Gram-negative bacilli.

Table 5.6. Number of non-infected quarters and bacterial species isolated from quarters one day after calving and four days after calving

	Treatment							
	DryFlex				Band-Aid			
	1 day after calving		4 days after calving		1 day after calving		4 days after calving	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Non-infected	94	107	95	100	103	109	100	99
Streptococcus uberis	19	15	11	10	15	8	16	8
Other Gram (+)ve, catalase (-)ve cocci	2	0	1	1	1	2	0	2
Coagulase-negative staphylococci	6	6	3	6	5	4	4	9
Coagulase-positive staphylococci	0	0	0	1	0	0	1	0
Gram-positive bacilli	17	8	14	11	5	10	13	8
Gram-negative bacilli	1	0	0	0	1	0	0	0
Contaminated	25	28	37	34	34	31	26	33
Missing	4	4	7	5	0	0	4	5
Total	168	168	168	168	164	164	164	164

5.3. DryFlex persistence on teats

The visible persistence of DryFlex after the single application at dry-off is illustrated in Figure 5.5. This figure shows that the teat seal was visible on 50% of the teat-ends for four days. DF persistence dropped to only 12% by day 9. The average number of days for which the teat-ends remained covered with DF was 5.2 for all quarters, with a range of 1 to 27 days. The teat seal persisted for longer in front quarters (average 7.3 days) than in rear quarters (average 3.2 days).

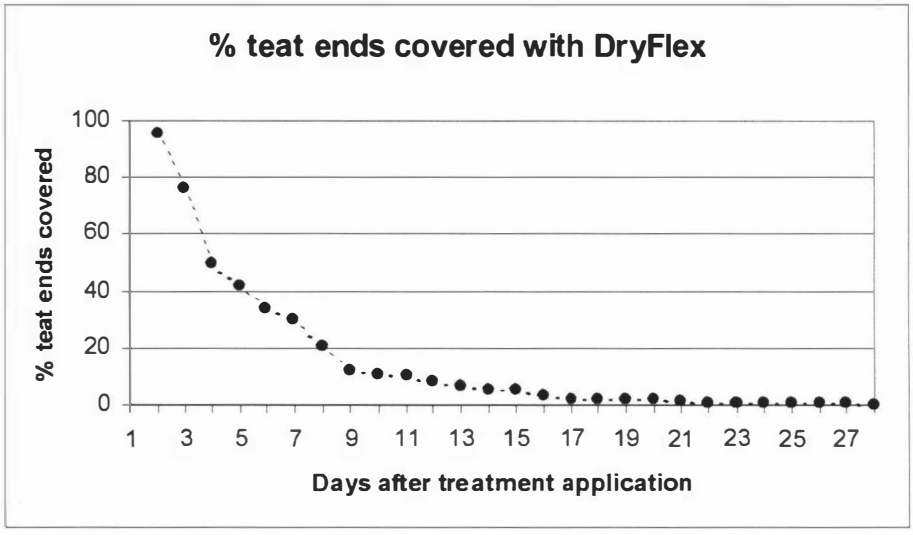

Figure 5.5. Proportion of quarters with DryFlex after a single application at dry-off

5.4. Pulsed-field gel electrophoresis

PFGE *SmaI* restriction fragments produced banding patterns consisting of 10-18 bands that ranged in size from approximately 48.5 to 700 kb pairs. The 19 isolates that were tested exhibited 14 distinct banding patterns. The banding pattern of three of these isolates was indistinguishable from that of the challenge organism. Eight isolates were closely related to the challenge organism and showed up to 4 bands that

were different. These isolates, together with the isolates showing the same banding pattern as the challenge strain, formed a well-defined cluster, with no chaining between its members and the remaining 8 isolates that were unrelated to the challenge strain (with 9 or more bands that were different). These were also unrelated to each other (data not shown). All 19 isolates revealed more than six bands that were different from the epidemiologically-unrelated control strain.

6. DISCUSSION

This study reports the first controlled experimental post-dry-off challenge trial of the efficacy of an external teat seal (DryFlex) and an octylcyanoacrylate (Band-Aid), in protecting against experimentally-induced mastitis caused by *Streptococcus uberis* after dry-off under dairy farming conditions in New Zealand.

Strep. uberis was selected as the challenge bacterium because it is the most important environmental pathogen causing mastitis during the dry period in New Zealand (Williamson *et al.*, 1995) and because it is relatively susceptible to treatment. No information was available from the scientific literature on the bacterial density needed to establish active intramammary infections by immersion of all the teats in a challenge suspension containing *Strep.uberis*. As expected, by standardizing the bacterial suspension according to a simple turbidity measurement, it was possible to obtain an inoculum of a concentration of the magnitude of 10^8 cfu/ml. In view of the high infection rate observed in the untreated quarters in the present study (47.2% and 36.8% for the DF and BA groups, respectively), this inoculum concentration could represent a benchmark for future studies using teat immersion as the method of exposure to *Strep. uberis*. Standardising the concentration of the bacterial suspension may not guarantee a successful challenge model every time. It may be important to use a very fresh bacterial suspension, as in this study. Furthermore, the ability of a strain to invade the teat is also a variable that should be taken into account when standardizing the inoculum-size since inter-strain variation has the potential to influence the success of the challenge.

Studies using natural exposure to pathogens and a post-calving efficacy assessment are difficult to design, prolonged, difficult to organise, expensive to run and prone to conflicting results. In addition, the incidence of post-calving mastitis can vary between herds and years and occasionally, the incidence might be low, resulting in underpowered studies. Lastly, as herds are dynamic populations, withdrawn cows could generate a significant lost-to-follow-up bias. For all of these reasons, such 'natural exposure experiments' are not suitable for commercial research. In this study, we developed and demonstrated the efficacy of a rapid and powerful method for the assessment of the antibacterial sealing effect of external teat seals against a standardised exposure to *Strep. uberis* by immersion of each teat in a broth. The advantages of the experimental model that we utilised are: a) it reduces the time needed to assess the barrier effect of the seal, from approximately two to three months (from dry-off to calving), to a few days; and 2) it significantly increases the number of mastitis events, enhancing statistical power for the comparison between treatments. Hence, the model that we developed and utilised is suitable for commercial research. The model used in this project is useful during development phases of the external teat seal. However, the ultimate assessment of efficacy of an external teat seal should be based on clinical trials with natural exposure to pathogens and should include post-calving efficacy assessment.

The experimental challenge model that we developed and utilised appeared to be a successful one to test the efficacy of the external teat seals. Indeed, the use of PFGE in a subset of isolates showed that 11 of 19 of the intramammary infections found between six and eleven days after dry-off (peak of infection) were caused by the challenge strain. PFGE analysis on all *Strep. uberis* isolates found after dry-off

would have provided a more complete representation of the occurrence of clinical mastitis caused either by the *Strep. uberis* challenge strain used in this study or by other *Strep. uberis* strains. This could not be done due to restrictions in budget.

In the present study, *Corynebacterium bovis* was highly prevalent in milk samples prior to dry-off. A high prevalence of *C. bovis* denotes poor use of teat disinfectants (Hillerton *et al.*, 1995; Anon., 1999). It is therefore likely that the teat disinfectant procedure used in this herd by automatic spraying was ineffectual in controlling the *C. bovis* colonising the teats in this herd

The infection status of quarters relating to the milk samples that were misplaced in the laboratory is available and only the information on the bacterial species causing the infections is missing. Therefore, this limitation does not influence the results and implications of the study, given that the main results were based on the occurrence and recognition of clinical mastitis and no bacteriological analysis was conducted on non-mastitic quarters. Consequently, the loss of these milk samples did not introduce any bias in the study. It is conceivable that the majority of the infections for which no bacterial species was available were caused by *Strep. uberis*, as indicated by the infections in the previous and following days, but this is unknown.

The use of DryFlex caused a significant decrease in the incidence of new intramammary infections in the early dry period compared with untreated quarters. In a previous study using the external teat seal based on tetrahydrofuran (DryFlex) under natural exposure, Timms (2001) reported lower infection levels after dry-off, with similar but smaller differences between control and treated quarters (13% and

5%, respectively) to those reported in the present study (47% and 20% for the control and external teat seal quarters, respectively). Both of these studies showed lower incidence of infection in the treated quarters; even though the overall incidence of infection was much higher in the present study with its experimental bacterial challenge.

Assessment of the efficacy of external teat seals in the prevention of new IMI needs to compare mastitis prevalences between treated and untreated quarters before and after calving. Timms (2001) reported a reduction in intramammary infection prevalences after calving subsequent to using the external teat seal pre-partum (see Table 2.3). Similarly, Young *et al.* (2005) found reductions of 23% ($p<0.5$) and 49% ($p<0.1$) in the incidence of new IMI in cows treated with DCT and the external teat seal at dry-off and cows treated with DCT and external teat seal pre-calving, respectively, in comparison with cows treated with DCT alone. The present study was designed to compare clinical mastitis incidence rates post-dry-off, because the post-calving assessment was confounded by the fact that quarters showing clinical mastitis post-dry-off were treated with antimicrobials. This was done to minimise disruption to the commercial herd in the following lactation.

In the present study, DF persisted for three days after application on 76% of teat-ends, which is lower than the 85% reported by Timms (2001). This could be due to the difference between the formulations of the teat seals used in the two trials or to the different environments: with cows kept in tie-stall barns in the previous study vs. cows kept at pasture in the present trial. The persistence of DF on teats after three days of application shown by Hayton and Bradley (2001) was also higher than was

observed in the present trial (>80% vs. 76%). However, in the organic study reported by Hayton and Bradley (2001) the persistence of DF on teats one week post-dry-off was 21%, a lower value than the 30% found in this study. This difference could be due to the different accommodation types used in the studies.

The Canadian study (Leslie *et al.*, 1999) found that teat-ends were covered for an average of 6.3 ± 0.1 days for all formulations, with a range of 0 to 15 days depending on the formulation. In the present trial, teat-ends remained covered with DF for an average of 5.2 days for all quarters, with a range of 1 to 27 days. The seal persisted for longer in front quarters than in rear quarters after dry off, in agreement with the UK data on organic herds (Hayton and Bradley, 2001).

The duration of the adherence of the external teat seal to the teat and the protection conferred to the teat orifice are critical factors in the performance of a teat seal. Earlier studies identified some factors that influence persistence, such as teat-end lesion score, teat length (Leslie *et al.*, 1999), milk yield and dry-cow accommodation type (Hayton and Bradley, 2001). The development of a formulation that persists for longer on teats of grazing cows would be of great benefit for the dairy industry.

The present study was performed in New Zealand, whereas previous studies were performed in the United States, United Kingdom, Canada and Argentina. The differences between New Zealand and the other countries in terms of housing, non-lactating cow management and calving management do not facilitate comparison of results from these studies.

In New Zealand, only a small proportion of herds treat all cows with antibiotics after dry off. Therefore, comparison of quarters that received an external teat seal with quarters that received no treatment at all is consistent with industry practice in this country, while also being of scientific merit.

To our knowledge, studies on the use of octylcyanoacrylate as a tool to prevent mastitis during the dry period of dairy cattle have not previously been reported. Thus, the present study is unique in that it evaluated the use of an octylcyanoacrylate, which is intended for use on minor cuts and scrapes of humans, on the prevention of mastitis in dairy cows after dry-off. In contrast to the DryFlex results, the use of an octylcyanoacrylate showed no association with a reduction in intramammary infections after dry-off when compared with quarters left untreated.

7. CONCLUSIONS

The experimental challenge model developed and used in this study appeared to be successful in generating intramammary infections during the dry period of dairy cows and proved a robust test of teat seals. The use of PFGE in a subset of isolates from post-dry-off samples was a useful tool to show that most of the clinical mastitis cases found at the peak of infection were due to the *Streptococcus uberis* strain used for the experimental challenge.

The application of DryFlex at the end of lactation was beneficial in the protection of quarters against mastitis in the early dry period caused by a bacterial challenge after dry-off. However, any effects on mastitis rates post-calving were confounded by the fact that quarters showing clinical mastitis post-dry-off were treated with antimicrobials. A teat seal of this type could be considered as a possibility for treatment of quarters at drying-off, since the data showed that DF was effective in giving short-term protection after treatment. Further work will be required to improve the persistence of the teat seal on teats. If persistence could be improved then the duration of protection would probably increase but it must also result in less infection after calving.

The octylcyanoacrylate used in the present study showed no benefit in reducing the incidence of mastitis due to bacterial challenge after dry-off.

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