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In vitro and *in vivo* studies on treatment
and prevention of bovine mastitis

A thesis presented in partial fulfilment of the requirements for the degree of
Philosophy Doctor
in
Veterinary Science

at Massey University, Palmerston North,
New Zealand.

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2011

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Abstract

Mastitis prevalence on dairy farms depends on the number of infected cows and the duration of each intramammary infection. Strategies aiming to influence these factors are the subject of research presented in this thesis.

Decreasing the duration of infection can be achieved by successfully treating infected quarters. Treatment of mastitis can occur during lactation or in the dry period. Treatment success is influenced by the concentration of antimicrobial achieved at the site of infection and the length of time it is present. The concentration of antimicrobial should exceed the relevant minimal inhibitory concentration. The susceptibility of mastitis-causing organisms varies among geographical areas and over time. New Zealand's susceptibility data demonstrated a high susceptibility to penicillin. A formulation containing this antimicrobial was administered to healthy lactating cows milked once or twice daily. The concentrations of penicillin in milk were above the minimal inhibitory concentrations for the entire inter-dosing interval. Doubling the number of treatments or milking once-a-day resulted in a significantly increased time above the minimal inhibitory concentrations.

The number of new infections is greatest during the early dry period in mature cows and in the pre-calving period in both heifers and mature cows. Pre-partum administration of delayed release antimicrobial formulations in heifers decreased the incidence of clinical mastitis and resulted in better reproductive performance, but not in increased milk production, when compared to control heifers. More effective prevention of new infections within the dry period was achieved by administering a novel teat sealant to mature cows when compared to a commercial teat sealant and untreated controls.

Strategies for shortening the duration of intramammary infections and decreasing the number of affected cows at the start of lactation investigated in this thesis should reduce the prevalence of mastitis on dairy farms in New Zealand.

KEY WORDS: Aetiology, Antibiotic, Antimicrobial, Challenge, Dry period, Experimental challenge, Heifers, Individual Cow Somatic Cell Count, Internal teat sealant, Mastitis, Milking frequency, Penicillin G, Reproductive performance, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, Susceptibility, Time Above the Minimal Inhibitory Concentrations, Udder, Withholding Period.

Acknowledgments

The PhD journey is long and extremely demanding. This thesis represents a multidisciplinary research effort and many people have contributed to its realisation. It is a very difficult task to remember everyone who took a part in helping me through this journey of a PhD candidacy.

I would like to say thanks to my Supervisors: Prof Norman B Williamson (Chief Supervisor), Prof Timothy J Parkinson (Internal Supervisor), Prof Ian J Tucker (External Supervisor) and Assoc Prof Nicolas Lopes-Villalobos (Internal Supervisor) for their guidance and support in this journey.

Thanks also to the co-authors in the various papers: Mohamed Abdalla, Alfredo Caicedo-Caldas, Alejandro (Alex) Grinberg, Richard Laven, Scott McDougall and Paul Rapnicki.

Massey University and particularly the Institute of Veterinary, Animal and Biomedical Sciences are thanked for employing me with financial support from *Bomac a company of Bayer Ltd.*

The experiments reported in this thesis would not have happened without financial support from *Bomac a company of Bayer Ltd* (formerly *Bomac Laboratories Ltd*) and I would like to express my sincere gratitude for their support.

I am indebted to all farm staff in the involved farms, staff at *Gribbles Veterinary Laboratories* Auckland, Christchurch, Dunedin, Hamilton, and Palmerston North, New Zealand and the *Veterinary Diagnostic Laboratory, University of Minnesota*, Minnesota, USA and *MilkTest NZ* (formerly *SAITL Dairy Laboratories*), Hamilton, New Zealand for their friendship, cooperation and interest in my research. Without their full support and cooperation the work reported in this thesis could not have been carried out.

In this thesis five experiments involved the use of dairy cows. For each individual experiment appropriate Animal Ethics Committee was granted by various committees. I would like to say thanks to the committee members for their understanding and approvals of the experiment designs.

It is not possible to list the names of everyone who contributed either directly or indirectly towards this work. Hence, I wish to express my sincere gratitude to all those who discussed matters related to this work and are not mentioned individually. The

anonymous reviewers of the manuscripts are thanked for their constructive criticism of the submitted work.

Some people who assisted me in the preparation of this thesis by taking part in some of the work, helping with agreements for funding, discussion, advice, statistical analysis or just being there when needed:

1. Past and present staff at *Bomac a company of Bayer Ltd*, Auckland, New Zealand: Fadil Al Alawi, Danielle Baxter, Ray Castle, Richard Emslie, Renee Hetherington, Wayne Leech, Connell McLaren, Don McLaren, Lina Ma, Rob Nottingham, Michael Syme, Warren Tully and Bruce Vautier.
2. Past and present staff at *Estendart Ltd*, Palmerston North, New Zealand: Alan Alexander, Hailey Baird, Rene Corner, Kara Eaton, Gilly Evans, Kathryn Hutchinson, Lina Yang, Jude Vautier and many casual staff.
3. Past staff at *Invoco – AgResearch*, Palmerston North: Jeremy Lind, Leonora Pearson and Brian Timms.
4. Past and present staff at *JL Vet Services*, Palmerston North: Jeremy Lind and many casual staff.
5. Past and present farm staff – John Allen, Wendy Allen, Hamish Doohan, Christine Finnegan, Conrad Maeke, Phil Martin and Grant Rudman.
6. Massey University, past and present staff at the *Agricultural Farm Services* – Louise Beazley, Natalie Butcher, Martin Chesterfield, Gareth Evans, Erin Hutchinson, Mark Lawrence, Natalia Martin and Byron Taylor.
7. Massey University, past and present staff at the Institute of Veterinary, Animal and Biomedical Sciences – Frazer Allen, Rukhshana Akhter, Hugh Blair, Andrea Coleman, Georgie Cowley, Gina de Nicolo, Sharron Hawira-Seanoa, Debbie Hill, Litty Kurian, Sue Leathwick, Gayle McKenna, Hamish Mack, Carol Orr, Rebecca Patisson, Quentin Roper, Kevin Stafford, Peter Wildbore and Dianna Willson.
8. Massey University, past and present staff at the *Research Management Services*: Nicola Carse, Mark Cleaver, Don Brown, Leith Hutton and Carolina Tate.
9. Massey University, Turitea campus, Library staff – Chris Good and Bruce White.
10. Massey University, *Large Animal Teaching Unit* – Liz Gillespie and Robin Whitson.
11. Massey University, past and present staff at the Veterinary Teaching hospital – Lesley England, Kevin Lawrence, Jenny Nixey, Alan Thatcher and Jenny Weston.

12. *University of Otago, School of Pharmacy*, past and present staff: Olaf Bork and Zimei Wu.
13. Duncan Hedderley from *The New Zealand Institute for Plant and Food Research Ltd*, Palmerston North.
14. Hassan Hussein from *Cognosco*, Morinsville, New Zealand.
15. Yuanxiang Shi, a visiting scholar from China.

Finally, my family and my dear wife, Ljubica (Bube) for putting up with me during this project.

Table of Contents

Abstract	I
Acknowledgments	III
List of Equations	XIII
List of Figures	XV
List of Tables	XIX
List of Abbreviations	XXV
1. General introduction to the thesis	3
1.1 Background and areas of research of the thesis	3
1.2 Aims of the thesis	10
1.3 Research objectives of the thesis	10
1.3.1 Part One	10
1.3.2 Part Two	11
1.3.3 Part Three	11
1.3.4 Part Four	11
1.4 References	12
Part one	15
2. Introduction to part one: Antimicrobial susceptibility	19
2.1 References	26
3. Culture results from 25,288 milk samples submitted to veterinary diagnostic laboratories from August 2003 to December 2006 in New Zealand	33
3.1 Abstract	33
3.2 Introduction	34
3.3 Materials and methods	35
3.3.1 Microbiological methods	35
3.3.2 Statistical analysis	36
3.4 Results	39
3.5 Discussion	43
3.6 Conclusion	46
3.7 Acknowledgments	46
3.8 References	46

4.	A descriptive analysis of the antimicrobial susceptibility of mastitis-causing bacteria isolated from samples submitted to commercial diagnostic laboratories in New Zealand (2003–2006)	55
4.1	Abstract	55
4.2	Introduction	56
4.3	Materials and methods	57
	4.3.1 <i>Criteria for selection of cases</i>	57
	4.3.2 <i>Microbiological methods</i>	58
	4.3.3 <i>Other records</i>	59
	4.3.4 <i>Statistical analysis</i>	59
4.4	Results	60
	4.4.1 <i>Antimicrobial susceptibility of streptococci</i>	61
	4.4.2 <i>Antimicrobial susceptibility of staphylococci</i>	64
	4.4.3 <i>Antimicrobial susceptibility of other bacterial species</i>	66
4.5	Discussion	66
4.6	Acknowledgements	77
4.7	References	77
5.	Susceptibility to antimicrobials of mastitis-causing <i>Staphylococcus aureus</i>, <i>Streptococcus uberis</i> and <i>Strep. dysgalactiae</i> from New Zealand and the USA as assessed by the disk diffusion test	83
5.1	Abstract	83
5.2	Introduction	84
5.3	Materials and methods	85
	5.3.1 <i>Statistical analysis</i>	87
5.4	Results	89
	5.4.1 <i>Level of susceptibility</i>	94
	<i>Susceptibility of Staphylococcus aureus</i>	96
	<i>Susceptibility of Streptococcus spp</i>	96
	5.4.2 <i>Zones of inhibition</i>	99
	5.4.3 <i>Discordant isolates</i>	104
5.5	Discussion	104
5.6	Conclusion	108
5.7	Acknowledgments	109
5.8	References	109

6.	Correlation of the antimicrobial susceptibility of <i>Staphylococcus aureus</i> and streptococci isolated from bovine milk samples collected in New Zealand when tested by the agar disk diffusion and broth microdilution methods	115
6.1	Abstract	115
6.2	Introduction	116
6.3	Materials and methods	117
6.4	Results	120
	6.4.1 Overall	120
	6.4.2 Ampicillin	123
	6.4.3 Cloxacillin	126
	6.4.4 Enrofloxacin	128
	6.4.5 Neomycin	130
	6.4.6 Oxytetracycline.....	132
	6.4.7 Penicillin	134
6.5	Discussion	136
6.6	Conclusion	139
6.7	Acknowledgments	139
6.8	References	139
	Part two	143
7.	Introduction to Part Two: Effects of milking frequency on pharmacokinetics of penicillin G administered by the intramammary route	147
7.1	References	153
8.	Milking frequency affects the penicillin G elimination times from milk, concentrations and recovery rate following intramammary administration to dairy cows	159
8.1	Abstract	159
8.2	Introduction	160
8.3	Materials and methods	161
	8.3.1 Experimental animals	161
	8.3.2 Treatment and procedures	162
	8.3.3 Statistical analysis	163
8.4	Results	166
	8.4.1 Elimination times	166
	8.4.2 Time above MIC	166

8.4.3	<i>Amount of drug recovered</i>	167
8.5	Discussion	168
8.6	Conclusion	172
8.7	Acknowledgments	172
8.8	References	172
Part three		177
9.	Introduction to Part Three: treatment of heifers for mastitis pre-calving	181
9.1	References	187
10.	Treatment before calving of heifers for mastitis improves their reproductive performance, but not their milk production	195
10.1	Abstract	195
10.2	Introduction	196
10.3	Materials and methods	197
10.3.1	<i>Procedures</i>	197
10.3.2	<i>Statistical Analysis</i>	198
10.4	Results	199
10.4.1	<i>Incidence of clinical mastitis</i>	199
10.4.2	<i>Prevalence of subclinical mastitis</i>	199
10.4.3	<i>Days-in-milk</i>	203
10.4.4	<i>Milk production</i>	203
10.4.5	<i>Reproductive performance</i>	204
10.5	Discussion	205
10.6	Conclusions	208
10.7	Acknowledgments	208
10.8	References	208
Part four		215
11.	Introduction to Part Four: antimicrobial teat sealant for use at drying off	219
11.1	References	224
12.	A preliminary evaluation of the efficacy of two novel internal teat sealant formulations against bacterial challenge in the early dry period	229
12.1	Abstract	229
12.2	Introduction	230
12.3	Materials and methods	230

12.3.1	<i>Cows and treatments administered</i>231
12.3.2	<i>Procedures</i>231
12.3.3	<i>Statistical analysis</i>239
12.4	Results240
12.4.1	<i>Length of dry period</i>240
12.4.2	<i>Palpation scores</i>240
12.4.3	<i>Clinical mastitis</i>242
12.4.4	<i>Genotyping isolates of <i>Streptococcus uberis</i> from clinical cases</i>242
12.4.5	<i>Intramammary infection</i>242
12.4.6	<i>Somatic cells</i>244
12.5	Discussion244
12.6	Acknowledgments246
12.7	References246
13.	Efficacy of a novel internal dry period teat sealant containing 0.5% chlorhexidine against experimental challenge with <i>Streptococcus uberis</i> in dairy cattle251
13.1	Abstract251
13.2	Introduction252
13.3	Materials and methods253
13.3.1	<i>Animals</i>253
13.3.2	<i>Treatment products and treatment administration</i>254
13.3.3	<i>Procedures</i>254
13.3.4	<i>Statistical analysis</i>257
13.4	Results260
13.4.1	<i>Dry period</i>260
13.4.2	<i>Udder palpation scores</i>260
13.4.3	<i>Clinical mastitis during the palpation period</i>261
13.4.4	<i>Milk culture results</i>262
13.5	Discussion264
13.6	Conclusion268
13.7	Acknowledgments268
13.8	References268

14.	General discussion275
14.1	Part One275
14.2	Part Two279
14.3	Part Three280
14.4	Part Four281
14.5	Further research needs identified283
14.6	References284
15.	List of references289
16.	Bibliography315

List of Equations

Equation 1-1 Calculations of the prevalence of mastitis on a dairy farm	3
Equation 4.1 Back-transforming of the model outputs	60

List of Figures

Figure 1.1. Incidence and prevalence of intramammary infections in a herd throughout the season when each infection is of a long duration. The prevalence at the moment of observation is high despite the low incidence	4
Figure 1.2. Incidence and prevalence of intramammary infections in a herd throughout the season when each infection is of a short duration. The prevalence at the moment of observation is low despite the high incidence	5
Figure 1.3. Incidence of intramammary infections through the lactational cycle provided no dry cow therapy is used	8
Figure 2.1. Trend in the number of herds and average herd size from 1974/75 to 2009/10	26
Figure 3.1. Origin of the milk samples submitted for culturing to five commercial laboratories in New Zealand from August 2003 to December 2006	38
Figure 3.2. Monthly isolates of <i>Staphylococcus aureus</i> and <i>Streptococcus uberis</i> as percentage of all samples submitted from August 2003 to December 2006 from winter to autumn	39
Figure 3.3. Monthly isolates of other commonly-isolated mastitis-causing organisms Zealand as percentage of all samples submitted from August 2003 to December 2006 from winter to autumn.	40
Figure 3.4. Percentage of isolates of <i>Streptococcus uberis</i> , <i>Staphylococcus aureus</i> by seasons	42
Figure 5.1 Agar disk diffusion and E-test of <i>Staphylococcus</i> isolate	88
Figure 5.2 Agar disk diffusion and E-test of streptococcal isolate	88
Figure 6.1. Schema of the graphic presentation of each antimicrobial/isolates of causative organism susceptibility testing outcome	120
Figure 6.2. Distribution of susceptibility results of isolates of <i>Staphylococcus aureus</i> or streptococci to ampicillin	125

Figure 6.3. Distribution of susceptibility results of isolates of <i>Staphylococcus aureus</i> or streptococci to cloxacillin	127
Figure 6.4. Distribution of susceptibility results of isolates of <i>Staphylococcus aureus</i> or streptococci to enrofloxacin	129
Figure 6.5. Distribution of susceptibility results of isolates of <i>Staphylococcus aureus</i> or streptococci to neomycin	131
Figure 6.6. Distribution of susceptibility results of isolates of <i>Staphylococcus aureus</i> or streptococci to oxytetracycline	133
Figure 6.7. Distribution of susceptibility results of isolates of <i>Staphylococcus aureus</i> or streptococci to penicillin	135
Figure 8.1. Treatment by the intramammary route using partial insertion technique	163
Figure 8.2. Milk samples for various analysis and reserves	165
Figure 8.3. <i>DeLaval</i> in-line samplers	165
Figure 8.4. Concentrations of procaine penicillin G in milk (mg/kg) in cows treated 3 times and milked once-a-day or twice daily and treated 6 times and milked twice daily	167
Figure 10.1. Moving average (10-daily) of the predicted geometric mean of individual cow somatic cell count in treated and control heifers in their first lactation	201
Figure 10.2. Percentage of treated heifers with high individual cow test-day somatic cell count (ICSCC \geq 200,000/mL) percentage of new infections (change of ICSCC from low to high) and percentage of cured cases (change of ICSCC form high to low) through their first lactation	202
Figure 10.3. Percentage of control heifers with high individual cow test-day somatic cell count (ICSCC \geq 200,000/mL) percentage of new infections (change of ICSCC from low to high) and percentage of cured cases (change of ICSCC form high to low) through their first lactation	203

Figure 10.4. Predicted milk volume using the method of Ali and Schaeffer (1987) in treated and control heifers during their first lactation204

Figure 11.1. Treatment design for the second challenge study220

Figure 12.1 X-ray picture of the location of the internal teat sealant post treatment with the sealant showing as a bright white area within the teat cavity234

Figure 12.2 X-raying of the teats after treatment in order to evaluate the position of the teat sealant as shown in Figure 12.1235

Figure 12.3. Average daily udder palpation score241

Figure 13.1. Survival analysis from treatment to incidence of clinical mastitis during the first 34 days after drying-off262

List of Tables

Table 2.1. Summary of the three general mechanisms of acquired resistance in pathogenic microorganisms	21
Table 2.2. Classification of efflux resistance mechanisms present in pathogenic microorganisms based on the spectrum of activity to various antimicrobial classes and transporter families	22
Table 2.3. Summary of the general mechanisms of intrinsic resistance in pathogenic microorganisms	23
Table 3.1. Counts of mastitis-causing organisms isolated from milk samples submitted to five commercial laboratories in New Zealand from August 2003 to December 2006 after reclassification	37
Table 3.2. Percentage (\pm standard error) of isolation of <i>Streptococcus uberis</i> by year, season and island from milk samples submitted to five commercial laboratories in New Zealand from August 2003 to December 2006	41
Table 3.3. Percentage (\pm standard error) of isolation of <i>Staphylococcus aureus</i> by year, season and island from milk samples submitted to five commercial laboratories in New Zealand from August 2003 to December 2006	41
Supplementary information - Table 3.4. Counts of mastitis-causing organisms isolated from milk samples submitted to five commercial laboratories in New Zealand from August 2003 to December 2006	48
Table 4.1. Number of tests for antimicrobial susceptibility of mastitis-causing bacteria isolated from samples submitted to five commercial laboratories in New Zealand over a 40-month period (2003–2006), categorised by region of origin of sample	58
Table 4.2. Number of tests for antimicrobial susceptibility, and percentage susceptibility, of seven mastitis-causing bacterial pathogens isolated in pure culture, each with >1,000 tests, and of 11 antimicrobials tested against >500 isolates from milk samples submitted to five commercial laboratories in New Zealand over a 40-month period (2003–2006)	62

Table 4.3. Number of tests and estimated percentage susceptibility, with 95% CI, to antimicrobials for isolates of <i>Streptococcus uberis</i> from milk samples submitted to five commercial laboratories in New Zealand over a 40-month period (2003–2006), adjusted for effect of year of testing, island of origin of sample, and the interaction of year and antimicrobial	63
Table 4.4. Number of tests and estimated percentage susceptibility, with 95% CI, to antimicrobials for isolates of <i>Staphylococcus aureus</i> from milk samples submitted to five commercial laboratories in New Zealand over a 40-month period (2003–2006), adjusted for effect of year of testing, island of origin of sample, and the interaction of year and antimicrobial	65
Table 4.5. List of available pharmaceutical products containing a minimum of one antimicrobial authorised for the treatment of bovine mastitis in New Zealand (2009), as indicated on the label’s recommendations	73
Table 5.1. Disk potency of antimicrobials used in the study (µg- micrograms)	87
Table 5.2. Susceptibility of isolates of <i>Staphylococcus aureus</i> and streptococci by country	90
Table 5.3. Susceptibility of streptococcal isolates by country	92
Table 5.4. Prevalence of susceptibility (mean ± SE) of <i>Staphylococcus aureus</i> and streptococci isolated from milk samples collected in New Zealand and the USA to a range of antimicrobials	95
Table 5.5. Prevalence of susceptibility (mean ± SE) of <i>Streptococcus uberis</i> and <i>Strep. dysgalactiae</i> isolated from milk samples collected in New Zealand and the USA to a range of antimicrobials	98
Table 5.6. Diameters of zones of inhibition (mean ± SE) for susceptible and resistant isolates of <i>Staphylococcus aureus</i> and streptococci isolated from milk samples collected in New Zealand and the USA	100
Table 5.7. Diameters of zones of inhibition (mean ± SE) for susceptible and resistant isolates of <i>Streptococcus uberis</i> and <i>Strep. dysgalactiae</i> isolated from milk samples collected in New Zealand and the USA	102

Table 6.1. Interpretive criteria for bacteria isolated from animals (if not stated otherwise based on the Clinical and Laboratory Standards Institute, 2008)	119
Table 6.2. Average sizes of inhibition at agar disk diffusion (mm \pm SE) compared to microdilution test results (μ g/mL) for various antimicrobial and causative organism combinations	122
Table 6.3. Correlation parameters for the zones of inhibition measured using the agar disk diffusion method with the MIC obtained by the broth microdilution method for various antimicrobial/causative organism combinations	124
Table 8.1. Elimination times of penicillin G from milk (means \pm SE) in cows under different milking frequency and treatment regime treated with Lactapen G by the intramammary route	166
Table 8.2. Amount of penicillin G recovered from milk of cows under different milking frequency and treatment regime treated with Lactapen G by the intramammary route	168
Table 9.1. Reported prevalence of intramammary infections in heifers before the first calving based on culture	182
Table 9.2. Prevalence of intramammary infections in heifers around the first calving.	183
Table 9.3. Rate of clinical mastitis in heifers around calving or during the first lactation.	185
Table 10.1. Changes in the percentage of heifers with high individual somatic cell counts High ICSCC; $SCC \geq 200,000/mL$) from low to high (New infections) and high to low (Cured infections) approximating rates of new infections and cures from subclinical mastitis and the percentage of high ICSCC on test-day in treated and control heifers during their first lactation	200
Table 10.2. Means \pm SE and differences of three reproductive parameters in treated and control heifers	204
Table 11.1. Summary of studies on the efficacy of teat sealants alone and their use in combination with other products	223

Table 12.1. Quarter and teat examination and palpation scores and description (criteria developed by KRP)	236
Table 12.2. Means and standard errors of the lengths of the dry period in days per group	240
Table 12.3. Palpation scores in the first 34 days after drying-off adjusted for the random effect of an individual cow	240
Table 12.4. Effect of treatment on palpation scores in the first 34 days after drying-off	241
Table 12.5. Incidence of clinical mastitis during the palpation period by treatment	242
Table 12.6. Prevalence of quarters with intramammary infection after calving	243
Table 12.7. Summary of the culture results in per cent (and numbers) after calving (D0 - day of calving; D4 - day 4 after calving) for all sampled quarters (including those treated for clinical mastitis during the palpation period)	243
Table 12.8. Means and their standard errors of the somatic cell scores (log of the somatic cell count divided by 1,000) among groups after calving	244
Table 13.1. The concentration of colony-forming units of a <i>Streptococcus uberis</i> S210 strain per millilitre in the challenge broth at different challenge days	255
Table 13.2. Quarter and teat examination and palpation scores and description	255
Table 13.3. Prevalence of infected or non-infected quarters in percent \pm standard errors among groups	259
Table 13.4. Effect of treatment on palpation scores in the first 34 days after drying-off	260
Table 13.5. Distribution of cases of clinical mastitis, the probability of a quarter being affected by clinical mastitis and probability of a quarter of being affected with clinical mastitis caused by the challenge organism in the first 34 days after drying-off	261

Table 13.6. Summary of the culture results in percent (and numbers) among groups263

Table 13.7. Means and 95% confidence intervals of the quarter level infection rate after calving264

List of abbreviations

Abbreviation	Meaning
ACVM	Agricultural Compounds and Veterinary Medicines Group (part of MAF New Zealand)
ATS	Anti-Infective-Containing Internal Teat Sealant
BAGG	Buffered Azide Glucose Glycerol broth
BMSCC	Bulk Milk Somatic Cell Count
CAMP	Christie–Atkins–Munch–Petersen test
CI	Confidence Interval
CLSI	Clinical and Laboratory Standards Institute
CMT	California Mastitis Test
CNS	Coagulase-Negative Staphylococci
DCT	Dry Cow Therapy
EMA	European Medicines Agency
EUCAST	European Committee on Antimicrobial Susceptibility Testing
I	Intermediate Susceptibility
ICSCC	Individual Cow Somatic Cell Count
IU	International Units
kg	Kilogramme
L	Litre
LF	Left Front
Ltd	Limited
µg	Microgram
mg	Milligram
mL	Millilitre
MIC	Minimal Inhibitory Concentration

List of Abbreviations continued

MRL	Maximum Residue Levels
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
NIRD	National Institute for Research in Dairying
OAD	Once-a-Day
PBP	Penicillin Binding Protein
PFGE	Pulse Field Gel Electrophoresis
R	Resistant
RR	Rear Right
S	Susceptible
SAMM	Seasonal Approach to Managing Mastitis
SAS	Statistical Analysis System
SCC	Somatic Cell Count
SCS	Somatic Cell Score
T>MIC	Time above the Minimal Inhibitory Concentrations
TD	Twice daily
WHP	Withholding period

Chapter 1

General Introduction

1. General introduction to the thesis

1.1 Background and areas of research of the thesis

Mastitis is one of the most common and economically important diseases of dairy cattle. Years of research have led the road to control of mastitis and mastitis management plans have been developed, starting with the original National Institute for Research in Dairying (NIRD) 5-point plan in the mid-1960s (Dodd *et al.* 1969). Control measures should constantly evolve in response to new knowledge based on research and practical findings. The aim of this thesis was to contribute to the validation and development of new mastitis control strategies.

In a practical environment veterinarians estimate the prevalence of mastitis on a dairy farm using Equation 1.1.

Equation 1-1 Calculations of the prevalence of mastitis on a dairy farm

$$\textit{Prevalence} = \textit{duration of each infection} * \textit{number of cows infected}$$

Thus, the prevalence of mastitis at any given time depends on the duration of each intramammary infection and the number of infected cows (Figures 1.1 and 1.2). Strategies that aim to influence the magnitude of these two factors are the subject of research presented in this thesis.

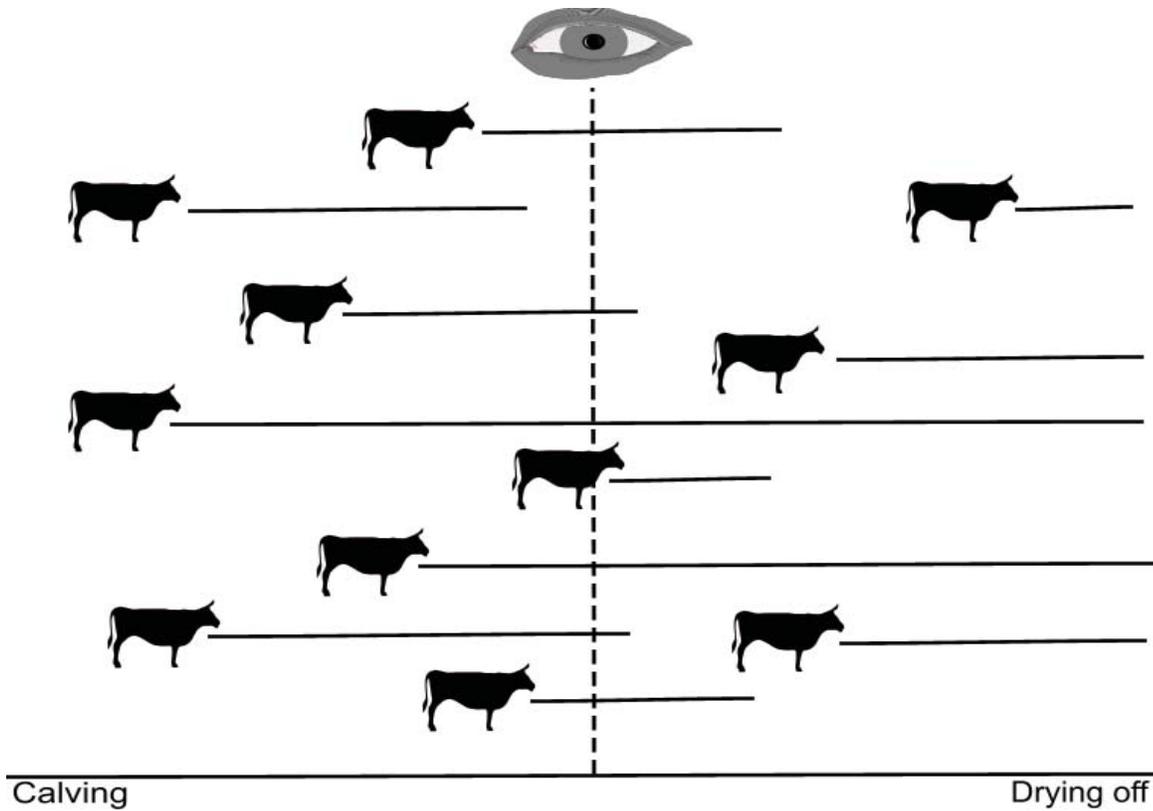


Figure 1.1. Incidence and prevalence of intramammary infections in a herd throughout the season when each infection is of a long duration. The prevalence at the moment of observation is high despite the low incidence (Adopted from Biggs A., *Mastitis in cattle*, Crowood Press, UK, 2009 and Pfeiffer D., *Veterinary epidemiology: An introduction*, Wiley-Blackwell, UK, 2010 – references not listed in the list of references)

The duration of each infection depends on the causative agent, the magnitude of response of udder defence mechanisms and age of the cow. The number of affected cows depends on the number of cows entering the new lactation infected and those getting infected during the season. The number of cows entering the season infected is a product of the persistent intramammary infections from the previous season and those acquired during the dry period.

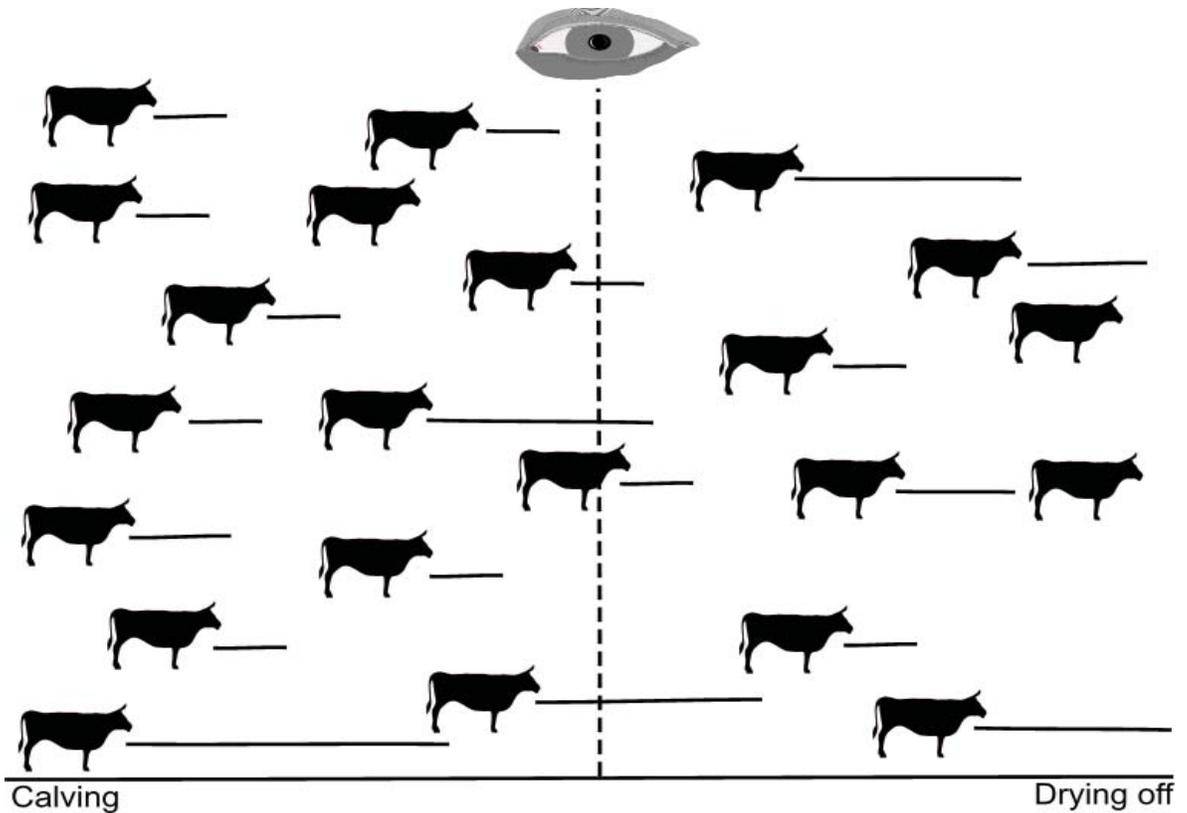


Figure 1.2. Incidence and prevalence of intramammary infections in a herd throughout the season when each infection is of a short duration. The prevalence at the moment of observation is low despite the high incidence (Adopted from Biggs A., *Mastitis in cattle*, Crowood Press, UK, 2009 and Pfeiffer D., *Veterinary epidemiology: An introduction*, Wiley-Blackwell, UK, 2010 – references not listed in the list of references)

Decreasing the duration of infection can be achieved by treating infected quarters. Treatment of mastitis can occur during lactation or in the dry period. Treatment of clinical cases of mastitis is a cornerstone of any mastitis management programme (Dodd *et al.* 1969; Brander 1975), including the ‘Smart SAMM’ in New Zealand that is currently under development.

More than 137 species of organisms have been implicated as causal agents of bovine mastitis (Watts 1988). Many bacteria, yeasts, viruses and fungi have been isolated from bovine mammary glands but only a small group of them cause elevated somatic cell counts and mastitis (Watts 1988; Malinowski *et al.* 2002; Wellenberg 2002). More than 90% of all new intra mammary infections are caused by a few mastitis causing organisms, namely, *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Streptococcus agalactiae* *Strep. dysgalactiae*, *Strep. uberis* and *Escherichia coli*. However,

there are many other species of organisms capable of causing mastitis and finding one of these causing mastitis may be highly significant for a particular dairy herd.

Most published surveys in peer-reviewed journals on the aetiology of bovine mastitis in New Zealand are over 40 years-old (Brookbanks 1966; Elliott *et al.* 1976a, 1976b). Since the 1970s, rapid genetic improvement in the milk production of dairy cows, changes in stocking rates and herd sizes (Anonymous, 2010) have occurred. Hence, results from studies undertaken at that time may no longer be pertinent to high-producing, modern cows with enhanced genetics. Furthermore, the implementation of mastitis management programmes and differing patterns of antimicrobial use have led to significant shifts in the aetiology of mastitis worldwide over this period (Erskine *et al.* 1988; Zadoks and Fitzpatrick 2009). These factors also have effects on the susceptibility of mastitis-causing organisms to various antimicrobials. Additionally, the susceptibility of organisms can be affected by the pattern of use of antimicrobials on dairy farms, in animals, humans and plants. Finally, the susceptibility of mastitis-causing organisms to antimicrobials may be changed over time due to the spread of resistant genes from resistant strains of the same species, and also from other members of the *phylum* or even between different *phyla*. Variations in practices can lead to differences seen between geographic locations from where samples for isolation of mastitis-causing organisms have been collected.

The prevalence of mastitis pathogens reported by Brookbanks (1966) and Elliott *et al.* (1976b) were 14 - 18% for *Strep. agalactiae*, 27 - 41% for *Staph. aureus*, 2 - 4% for *Strep. dysgalactiae*, <3.0% for *Strep. uberis*, and 25% for coagulase-negative staphylococci. The results of more recent clinical studies indicate that the relative prevalence of *Strep. agalactiae* and *Staph. aureus* have fallen, and that *Strep. uberis* is the most important cause of bovine mastitis in New Zealand. An intervention study on clinical mastitis undertaken in the South Auckland region (McDougall, 2003) reported that 56% of cases were *Strep. uberis*, 8% coagulase-negative staphylococci, 3% coliforms, 3% *Strep. dysgalactiae* and 2% *Staph. aureus*. A multicentre study of clinical mastitis, across both islands reported a different isolation pattern. In that, 32% of cases were due to *Strep. uberis*, 16.5% to *Staph. aureus*, 6% to *Strep. dysgalactiae* and 5.5% to coagulase-negative staphylococci (McDougall *et al.* 2007). A survey of clinical cases on 14 farms in Northland (Petrovski *et al.* 2009) reported another different pattern, with *Staph. aureus* being isolated from 24% of cases and *Strep. uberis* from 23% while no other organism was isolated from >3% of cases.

The success of treatment is influenced by the concentration and the length of time of the antimicrobial achieved at the site of infection (Ziv 1980; Craig 1998; Toutain 2003). The concentration of antimicrobial should exceed the minimal inhibitory concentrations (MICs) for a sufficient length of time at the site of infection. The susceptibility of mastitis-causing organisms varies among geographical areas and over time. Therefore, New Zealand-specific and current data were required. Part One of the thesis addresses this requirement.

The concentrations of an antimicrobial achieved in milk and the elimination times vary between products (Schipper 1955; Ullberg *et al.* 1958a, 1958b; Uvarov 1960; Funke 1961; Hogh and Rasmussen 1961; Rasmussen 1964; Uvarov 1969) and are affected by the treatment and milking regime (Knappstein *et al.* 2003; Stockler *et al.* 2009). Usual farming practice in New Zealand is milking cows twice daily. However, a number of farms are milking the cows once-a-day throughout the season. In early lactation, through the mating period, in late lactation and before drying-off some farmers with the usual twice daily milking switch the whole herd or a part of the herd to once-a-day milking. This is a common procedure for cows with a low body condition score, young cows and so-called at-risk-cows (*i.e.* cows that had a difficult calving, twins, retained fetal membranes and have been ‘downer cows’). Furthermore, once-a-day milking is a common practice for cows with mastitis during treatment and the withholding period, particularly in large herds. This milking frequency can also be used as one of the management procedures to deal with restricted feed availability. The effects of once-a-day milking on the concentrations of penicillin achieved in milks of treated cows, amounts of recovered drug and elimination times compared to twice daily milking are unknown. Part Two of the thesis addresses this issue.

The importance of the dry period (*i.e.* the time between the last milking of one lactation and calving at the start of the next) in the epidemiology and control of bovine mastitis has been elaborated in numerous reviews (Neave *et al.* 1950; Eberhart 1986; Dingwell *et al.* 2003a; Bradley and Green 2004). There is no intention to provide another review on this issue but to briefly describe the important factors during the dry period that affect susceptibility to new intramammary infections and, therefore, influence the number of cows entering the lactation already infected.

During the dry period, the mammary gland undergoes a series of changes rendering it susceptible to new intramammary infections. The mammary gland is particularly

susceptible to new intramammary infections during the early and late dry periods, correlating with involution and colostrogenesis, respectively (Neave *et al.* 1950; Cousins *et al.* 1980; Funk *et al.* 1982; Eberhart 1986; Dingwell *et al.* 2002; Green *et al.* 2002). The third stage of the dry period, the steady state, is a period when the mammary gland is refractory to new intramammary infections (Figure 1.3).

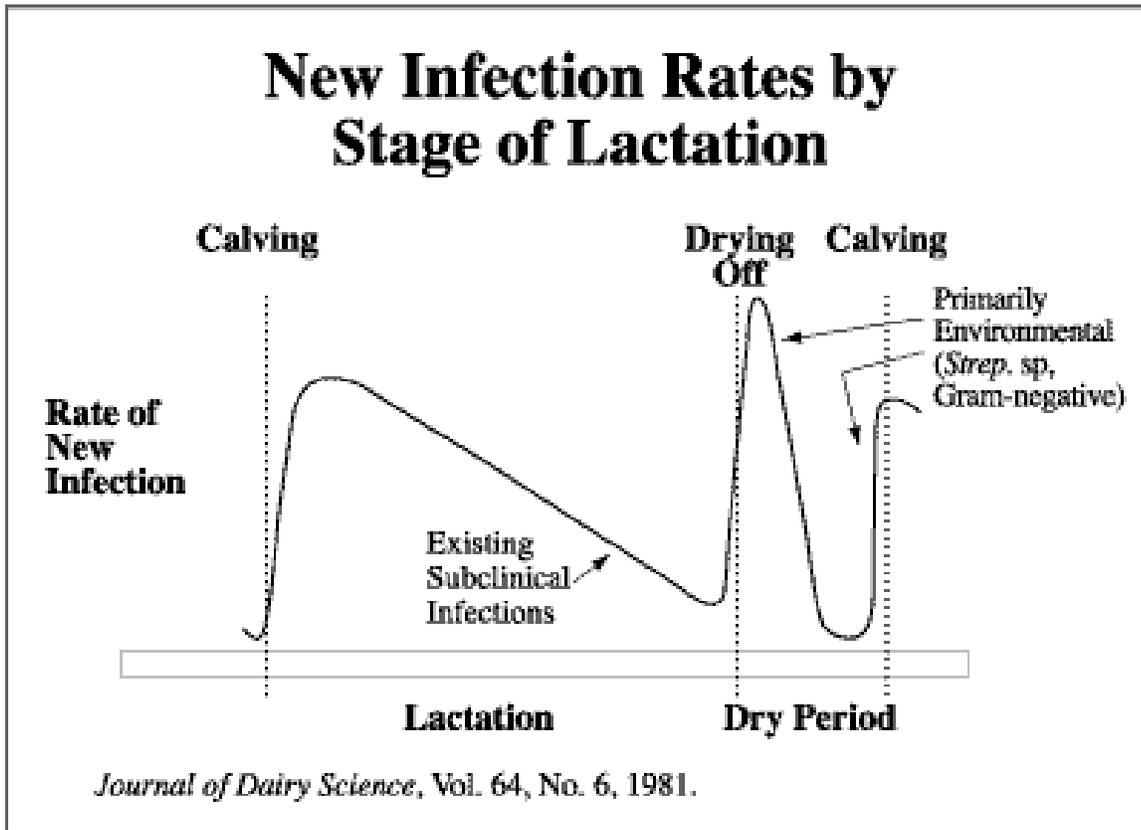


Figure 1.3. Incidence of intramammary infections through the lactational cycle provided no dry cow therapy is used (adapted by labelling from: Natzke, *Journal of Dairy Science*, 64: 1431-42, 1981)

Intramammary infections during the dry period may persist from the previous lactation or new infections may be acquired during the dry period (Cousins *et al.* 1980; Funk *et al.* 1982; Crispie *et al.* 2004). It is estimated that more than 50% of newly acquired intramammary infections in the early dry period persist into the next lactation if not eliminated by appropriate treatment (Berry and Hillerton 2002). New intramammary infections occurring in the dry period contribute in a major way to the increasing number of intramammary infections in each successive lactation.

A major risk factor allowing the invasion of mastitis causing organisms into the mammary gland during the dry period is the delayed formation of a competent keratin plug in the teat canal in the early dry period (Comalli *et al.* 1984; Bright *et al.* 1990; Woolford *et al.* 1998; Dingwell *et al.* 2003a; Dingwell *et al.* 2004). The keratin plug forms naturally in the teat canal after drying-off and appears to be a major defensive mechanism against infection (Bright *et al.* 1990; Woolford *et al.* 1998; Crispie *et al.* 2004) preventing the entry of causative organisms into the gland. Teats which formed a keratin plug soon after drying-off became classified as “closed” and rarely, if ever, became infected during the dry period (Woolford *et al.* 1998). Cows producing less milk at drying off had fewer open teats two and three weeks later than mid and high yielding cows (Odensten *et al.* 2007). A similar finding was reported by Dingwell *et al.* (2004). This may become important to the New Zealand dairy industry due to the recent movement toward intensification and provision of increased levels of supplemental food, rather than pasture only. A delay or absence in formation of the keratin plug is associated with an increased risk of new intramammary infections as 97% of these occurred in open quarters in a New Zealand study (Williamson *et al.* 1995). In a USA-based study, a 1.8 times increase in the risk of new intramammary infections occurred in quarters that remained open or had cracked teat-ends (Dingwell *et al.* 2003b).

Traditionally, heifers (two-year-old primiparous cattle) have been regarded as being free of mastitis as new introductions to a herd. In the last 2-3 decades this belief has been seriously challenged. Numerous studies have confirmed that the prevalence of intramammary infections in heifers may be high (Fox *et al.* 1995; Nickerson *et al.* 1995; Parker *et al.* 2007a; Parker *et al.* 2007b). The same is applicable for early post-calving clinical mastitis in heifers (Svensson *et al.* 2006; Compton *et al.* 2007; Parker *et al.* 2007a; Parker *et al.* 2007b).

Therefore, preventing the acquisition of new intramammary infections in the pre-calving period in heifers (rising 2-year-old primiparous cows) and during the dry period in mature cows is an important step in lowering the prevalence of mastitis on a dairy farm. In this thesis two different management measures involving treatment of heifers or mature cows will be addressed. The effect of administration of a delayed release antimicrobial-containing intramammary product in heifers a few weeks before calving on the incidence rate of clinical mastitis are addressed in Part Three of the thesis. In mature cows the effects of the administration of chlorhexidine-containing internal teat

sealant at the last milking of the season on the prevalence of intramammary infections at calving are addressed in Part Four of the thesis.

1.2 Aims of the thesis

The aim of the thesis was to address some strategies to influence the prevalence of mastitis on dairy farms by shortening the duration of intramammary infections using appropriately chosen treatment and decreasing the number of affected cows at the start of lactation in heifers and mature cows.

1.3 Research objectives of the thesis

Specific objectives of each Part are specified in the Chapters. The study objectives for each part are presented below.

1.3.1 Part One

- Descriptive analysis of the occurrence of mastitis-causing organisms from the cultures of milk samples submitted to a group of veterinary diagnostic laboratories across New Zealand from August 2003 to December 2006.
- Descriptive analysis of the antimicrobial susceptibility of mastitis-causing bacteria isolated from milk samples submitted to a group of veterinary diagnostic laboratories across New Zealand from August 2003 to December 2006.
- Assess and compare the susceptibility to selected antimicrobials of common mastitis-causing organisms isolated from milk samples collected in New Zealand or the USA, using disk diffusion tests.
- Challenge the assumption that testing a class representative or susceptibility at a genus level is sufficient for the three common mastitis-causing organisms.
- Assess the inter-test diagnostic agreement of the categorical interpretation criteria of results of agar disk diffusion and broth microdilution antimicrobial susceptibility testing methods, based on pre-set breakpoints.
- Evaluate the correlation between the sizes of zones of inhibition and MIC levels for isolates of *Staphylococcus aureus* and streptococci isolated from bovine milk samples collected in New Zealand.

1.3.2 Part Two

- Determine the elimination times from milk of an intramammary mastitis product containing penicillin G in cows milked at normal or reduced milking frequency.
- Determine the effect of the milking frequency and treatment regime on the persistence of an effective concentration of penicillin G in milk.
- Determine the effect of the milking frequency and treatment regime on the recovery of penicillin G in milk.

1.3.3 Part Three

- Determine the effect of treatment with a novel product intended for use in heifers pre-calving on the cumulative incidence of clinical mastitis, prevalence of subclinical mastitis, milk production and reproductive performance.

1.3.4 Part Four

- Compare the efficacy of two investigational teat sealants containing chlorhexidine with a commercial teat sealant not containing an antimicrobial agent and with untreated controls at cow-level-treatment against an experimental microbial challenge.
- Compare the efficacy of a teat sealant containing chlorhexidine with a commercial teat sealant not containing an antimicrobial agent and with untreated controls with within-cow control of both sealants against an experimental microbial challenge.

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Part 1

Antimicrobial susceptibility

Chapter 2

Introduction to Part One: Antimicrobial susceptibility

2. Introduction to part one: Antimicrobial susceptibility

In Part One of the thesis, surveys of the culture results and susceptibilities of bacterial isolates from milk samples submitted to a group of five diagnostic veterinary laboratories over a period of three and a half years are reported. The surveys were complemented with *in vitro* studies on the susceptibilities of three major mastitis-causing organisms (*Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Strep. uberis*) from New Zealand and the USA. Finally, the correlation between the results obtained by agar disk diffusion and broth microdilution methods was challenged. This work was done because the current knowledge on the antimicrobial susceptibility of mastitis causing organisms in New Zealand is limited.

An important step in the prevention of the development and dissemination of antimicrobial resistance is antimicrobial stewardship (Shlaes *et al.* 1997; Guillemot 1999; Courvalin 2005; Owens Jr and Ambrose 2007; Prescott 2008). Antimicrobial stewardship is defined as a process of reducing the development, maintenance and dissemination of resistance through reducing inappropriate and excessive use of antimicrobials coupled with optimal selection of antimicrobials, their dose and duration of treatment that will result in the best clinical outcomes (Owens Jr and Ambrose 2007; Prescott 2008). The Dairy Cattle Veterinarians Branch of the New Zealand Veterinary Association (DCV) has published guidelines for the use of antimicrobials in the 'New Zealand Dairy Cattle Formulary' (2008). The Guidelines clearly state recommended antimicrobial treatments for clinical mastitis cases and dry cow therapy.

To aid the process of development proper antimicrobial stewardship for the use of antimicrobials in treating bovine mastitis, better surveillance of resistance in mastitis-causing organisms and other animal pathogens is essential (Shlaes *et al.* 1997; Davison *et al.* 2000; Gnanou and Sanders 2000; Jorgensen 2004). Mastitis is the main reason for using antimicrobials in dairy cattle (Call *et al.* 2008). Therefore, the surveillance of resistance in mastitis-causing organisms is highly important. The value of regional, national and international surveillance data on resistance lies not in the use of these data to guide treatment for individual cases, but in the illustration of antimicrobial resistance trends that may require attention.

The volume of information concerning antimicrobial resistance is large. It is beyond the scope of this Chapter to review all of this literature. This Chapter is limited to the

microorganisms and antimicrobials that are currently of interest in the context of bovine mastitis.

During the treatment of infectious diseases, including bovine mastitis, resistance to antimicrobials can be encountered. However, it is important to recognise that antimicrobial resistance in clinical practice is only one cause of failure of antimicrobial treatment. Hence, its significance may be overemphasised. For example, the main reasons for clinical failure may be governed by the bioavailability of an antimicrobial at the site of infection (Drusano 2003; Melchior *et al.* 2006; Wright 2007) rather than the response of the microorganisms to the antimicrobial.

Antimicrobials used in veterinary medicine, including for treatment of mastitis, are largely the same as those used in the human medicine. An important exception for the treatment of mastitis in New Zealand is the use of tylosin, a macrolide. Therefore, much knowledge of antimicrobial resistance from human medicine is transferable to veterinary medicine.

Resistance is the temporary or permanent ability of an organism and its progeny to remain viable and/or multiply under conditions that would destroy or inhibit other members of the species (Schwarz and Chaslus-Dancla 2001; Cloete 2003). For microbial pathogens, resistance is encountered when members of a particular strain are not susceptible to concentrations of antimicrobials used in clinical practice (Gnanou and Sanders 2000; Cloete 2003).

Microorganisms have three general mechanisms of resistance to antimicrobials, namely: alteration of the antimicrobial target in the microbial cell, destruction or modification of the antimicrobial compound and prevention of the accumulation of the antimicrobial in the cell (Table 2.1). Alteration of the target site in the microbial cells, either through a synthesis of a resistant target or a resistant metabolic pathway is a common mechanism of resistance (Shlaes *et al.* 1997; Jeljaszewicz *et al.* 2000; McKeegan *et al.* 2002; Lambert 2005; Yoneyama and Katsumata 2006). Destruction or modification of an antimicrobial compound is usually mediated through intra- or extra-cellular enzymes (Shlaes *et al.* 1997). Enzymatic inactivation of antimicrobials in pathogenic microorganisms is probably acquired from other genera, including antimicrobial-producing microorganisms. Prevention of accumulation in the cell can be achieved either by preventing the uptake of the antimicrobial or by a rapid elimination from the cell (efflux pumps) (Shlaes *et al.* 1997; McKeegan *et al.* 2002; Yoneyama and Katsumata 2006;

Delcour 2009). Prevention of uptake of antimicrobials by pathogenic microorganisms involves alterations in the cell wall or cell membrane.

Table 2.1. Summary of the three general mechanisms of acquired resistance in pathogenic microorganisms

Mechanism of resistance	Examples	Antimicrobial class
Alteration of the target site	<ul style="list-style-type: none"> • alteration of penicillin binding proteins (PBPs) • methylation of adenine residues in 23S rRNA • modification of DNA-gyrase • alternative metabolic pathways not inhibited by antimicrobial compounds 	<ul style="list-style-type: none"> • beta-lactams • macrolides • fluoroquinolones • sulphonamides and trimethoprim
Destruction or modification of an antimicrobial compound	<ul style="list-style-type: none"> • beta-lactamases • intracellular enzymes • extracellular enzymes 	<ul style="list-style-type: none"> • beta-lactams • macrolides • aminoglycosides • other natural antimicrobial compounds
Prevention of accumulation in the cell	<ul style="list-style-type: none"> • porins • formation of biofilms • modification of DNA-gyrase • efflux pumps 	<ul style="list-style-type: none"> • beta-lactams • tetracyclines • fluoroquinolones

Efflux pumps (Table 2.2) limit the intracellular accumulation of cytotoxic compounds, including antimicrobials (Lyon and Skurray 1987; Ouellette and Kündig 1997; Nikaido 1998; McKeegan *et al.* 2002; Marquez 2005; Poole and Lomovskaya 2006; Alekshun and Levy 2007; Stavri *et al.* 2007; Wright 2007).

Table 2.2. Classification of efflux resistance mechanisms present in pathogenic microorganisms based on the spectrum of activity to various antimicrobial classes and transporter families

Spectrum of activity		Confers resistance to	Antimicrobial class affected
	Narrow spectrum	Single antimicrobial class	Tetracyclines
	Broad spectrum	Multi-drug resistance	
Efflux transporter families	H ⁺	MF (major facilitator)	
		RND (resistance nodulation and cell division)	
		SMR (small[or staphylococci] multi-drug resistance)	
	MATE (multi-drug and toxic compounds extrusion)		
	ATP	ABC (ATP-binding cassette)	

Antimicrobial resistance of microorganisms can be either intrinsic (passive; natural; innate) or acquired (active; inherent; adaptive).

Intrinsic resistance is a consequence of general adaptive processes that are not necessarily linked to a given class of antimicrobials (Wright 2005; Stavri *et al.* 2007; Bockstael and Van Aerschot 2009). This is better considered as insensitivity than resistance. Intrinsic resistance is usually species-related, not strain-related. The mechanisms of intrinsic resistance are presented in Table 2.3. Treatment of mastitis caused by organisms possessing intrinsic resistance is often very difficult. For example, the eradication of microorganisms associated with biofilms, such as *Staph. aureus* chronic mastitis (Melchior *et al.* 2006) often requires concentrations of antimicrobials hundreds or thousands of times higher than those required to kill planktonic (sessile) forms (Mah and O'Toole 2001; Melchior *et al.* 2006).

Table 2.3. Summary of the general mechanisms of intrinsic resistance in pathogenic microorganisms

Mechanism of resistance	Examples	Antimicrobial class affected
Low membrane permeability (Cohen and Tartasky 1997; McKeegan <i>et al.</i> 2002; Martínez-Martínez 2008; Delcour 2009)	<ul style="list-style-type: none"> • aminoglycosides unable to penetrate cells of enterococci • beta-lactams unable to penetrate cells of <i>Mycobacteria</i> • outer membrane of Gram-negative bacteria 	<ul style="list-style-type: none"> • aminoglycosides • beta-lactams
Chromosomally-encoded enzymes/genes (Cohen and Tartasky 1997; Davies and Davies 2010)	<ul style="list-style-type: none"> • antimicrobial-producing <i>Actinomycetes</i> or soil bacteria that degrade antimicrobials 	<ul style="list-style-type: none"> • all
Absence of an uptake transport system for the antimicrobial (Cohen and Tartasky 1997)	<ul style="list-style-type: none"> • low affinity penicillin binding proteins (PBP) in enterococci 	<ul style="list-style-type: none"> • beta-lactams
Absence of the target or reaction required for killing effect of an antimicrobial (Mateu and Martin 2001)	<ul style="list-style-type: none"> • absence of cell wall in <i>Mycoplasma</i> spp 	<ul style="list-style-type: none"> • penicillin
Formation of biofilms (Mah and O'Toole 2001; Melchior <i>et al.</i> 2006)	<ul style="list-style-type: none"> • Non-permeability (Neu 1982; Mah and O'Toole 2001) • Induction of biofilm-specific phenotype (Xu <i>et al.</i> 2000; Mah and O'Toole 2001) • Changes in metabolism (Xu <i>et al.</i> 2000; Stewart and William Costerton 2001) • Quorum sensing mechanisms (Mah and O'Toole 2001) • Changes in multiplication patterns (Mah and O'Toole 2001; Stewart and William Costerton 2001) • Expression of general stress response (Mah and O'Toole 2001) • Spatial heterogeneity (Xu <i>et al.</i> 2000) • Spore-like state of the microorganisms (Stewart and William Costerton 2001). 	<ul style="list-style-type: none"> • all

Acquired resistance is the result of a specific selective pressure and development of a survival strategy against a particular antimicrobials or classes of antimicrobials (Wright 2005; Bockstael and Van Aerschot 2009). This strategy selects microorganisms from a susceptible population to become resistant to a particular antimicrobial. Acquired resistance accounts for most of the resistance problems currently encountered in human and veterinary medicine. It is a strain-related characteristic. Acquired resistance can be developed by mutation or horizontal gene transfer (Boerlin and Reid-Smith 2008). Acquired resistance developed by mutation spreads vertically (Berger-Bächli 2002), e.g. methicillin-resistant *Staph. aureus* (MRSA). This organism has acquired the *mecA* gene from coagulase-negative staphylococci and has continued to spread it to their progeny worldwide (Cohn and Middleton 2009; Leclercq 2009). Therefore, due to the horizontal transfer of the mechanism of resistance, the genome of microorganisms of a strain within the species that have developed resistance by mutation is often genetically related worldwide.

Horizontal transfer of genes can occur within or between species (Courvalin 1994; Berger-Bächli 2002; Rowe-Magnus *et al.* 2002; Martel *et al.* 2005; Liñares *et al.* 2010) with the possibility of *trans-phylum* exchange even between Gram-negative and Gram-positive microorganisms (Neely and Holder 1999; Davison *et al.* 2000; Rowe-Magnus *et al.* 2002). Antimicrobial resistance can be also exchanged between microorganisms of various hosts, e.g. human and porcine streptococci and *vice versa* (Martel *et al.* 2005), poultry and human staphylococci (Teuber 2001), enterococci from bovine milk and human intestinal flora (Baumgartner 2001), streptococci from milk and human urogenital flora (Brown and Roberts 1991). This possibility has led to an increased scrutiny of antimicrobial use because of the real or perceived threats to human health.

The level of resistance differs between countries depending on the patterns of antimicrobial use (Gold and Moellering 1996; Franklin 1999; Aarestrup *et al.* 2002; Johnsen *et al.* 2009). The level of resistance is also affected (in humans) by the season and prescribing patterns, being lowest during the months of low use (Johnsen *et al.* 2009). Translating this to bovine mastitis under New Zealand seasonal calving conditions should result in a lower prevalence of resistance in the late winter, after a few months of no antimicrobial use (except for dry cow therapy; DCT). However, it is also possible that only resistant strains will survive inside the dry udder being treated with DCT.

The transfer of resistance from animal to human pathogens or *vice versa* is possible. Furthermore, the resistance mechanisms of commensal flora can be easily transferred to the pathogenic microbial population. The role of antimicrobial use in food producing animals and agriculture in the development of resistance and maintenance of antimicrobial resistance in human pathogens is still under debate. The exchange of genes between microorganisms of various hosts cannot be used as evidence of the importance of the development of resistance in animals or plants as a risk factor for resistance in human pathogens. The association is yet to be confirmed *in vivo* (Phillips *et al.* 2004; Cox Jr *et al.* 2007). The fundamental concern over antimicrobial use in food producing animals arises from the potential selection of resistant microorganisms on farms being transferred to humans *via* direct contact or indirectly (e.g. ingestion of contaminated food and water). Phillips *et al.* (2004) reviewed the correlation between the use of a specific antimicrobial class in animals and the resistance patterns in human pathogens and reported that there was no correlation. In contrast, a high correlation between the use of a specific antimicrobial class in humans and the patterns of resistance in human pathogens has been reported (Fujita *et al.* 1994; Seppälä *et al.* 1997; Kahlmeter 2003). However, the absence of proof should not be understood as an absence of a risk. Therefore, the control of resistance must rely on the collaboration of veterinary, human and phytomedicine. A thorough understanding of mechanisms of resistance is a pre-requisite for successful implementation of methodologies to reduce existing resistance and to inhibit the speed of development of new resistance. Additionally, the prescribing and use of antimicrobials in human and veterinary medicine must be based on sound knowledge of epidemiology, pharmacokinetics and pharmacodynamics.

Historically, the development and dissemination of resistance was important for nosocomial infections in human patients, but lately it is becoming a problem for the community (Alanis 2005). For veterinarians dealing with dairy cattle this may be translated to farm-specific strains. The current trend of increasing herd size (Figure 2.1; Anonymous 2006, 2010) increases the potential for resistance due to more closer contacts between cows.

Therefore, to estimate the current prevalence of resistance in common mastitis-causing organisms, a series of surveys and studies were carried out and are reported in Chapters 3 to 6. The results were compared to previous reports. This approach allows for estimation of the effect of modern dairy farming on the development of resistance.

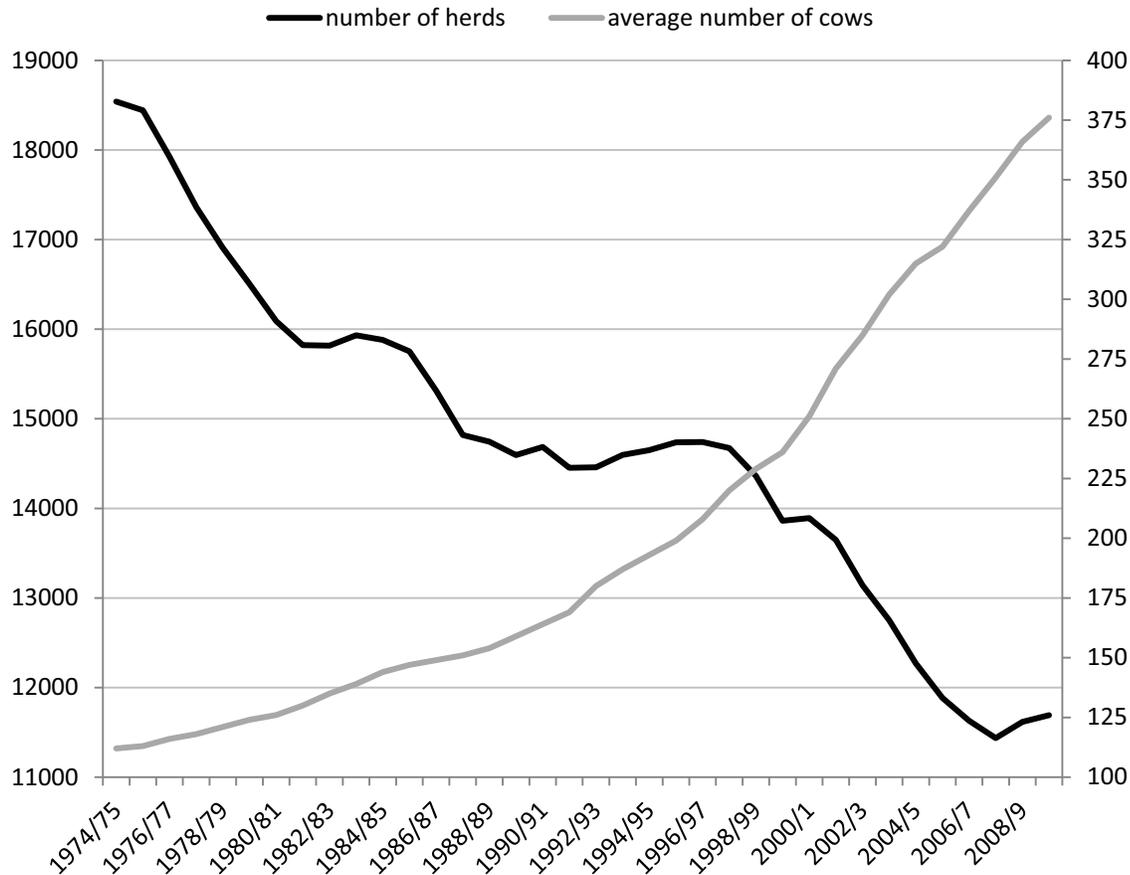


Figure 2.1. Trend in the number of herds and average herd size from 1974/75 to 2009/10 (data from Dairy Statistics 2009/10)

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Chapter 3

Culture results from 25,288 milk samples submitted to veterinary diagnostic laboratories from August 2003 to December 2006 in New Zealand

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Publication details:

Petrovski, K. R., Williamson, N. B., Lopez-Villalobos, N., Parkinson, T. J., & Tucker, I. G. Culture results from milk samples submitted to veterinary diagnostic laboratories from August 2003 to December 2006 in New Zealand. *New Zealand Veterinary Journal*, 59(6), 317-322. doi: 10.1080/00480169.2011.610286, 2011

Chapter 4

A descriptive analysis of the antimicrobial susceptibility of mastitis-causing bacteria isolated from samples submitted to commercial diagnostic laboratories in New Zealand (2003–2006)

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Publication details:

Petrovski K, Laven R, Lopez-Villalobos N. A descriptive analysis of the antimicrobial susceptibility of mastitis-causing bacteria isolated from samples submitted to commercial diagnostic laboratories in New Zealand (2003-2006). *New Zealand Veterinary Journal* 59, 59 - 66, 2011

Chapter 5

Susceptibility to antimicrobials of mastitis-causing *Staphylococcus aureus*, *Streptococcus uberis* and *Strep. dysgalactiae* from New Zealand and the USA as assessed by the disk diffusion test

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5. Susceptibility to antimicrobials of mastitis-causing *Staphylococcus aureus*, *Streptococcus uberis* and *Strep. dysgalactiae* from New Zealand and the USA as assessed by the disk diffusion test

5.1 Abstract

AIMS: Antimicrobial susceptibility of 182 *Staphylococcus aureus*, 126 *Streptococcus uberis* and 89 *Strep. dysgalactiae* that were isolated from milk samples collected in New Zealand (107, 106 and 41) and the USA (75, 20 and 48) was assessed using the disk diffusion test following the guidelines of the Clinical Laboratory Standards Institute (CLSI).

METHODS: Isolates were tested against the following antimicrobials: amoxicillin, amoxicillin/clavulanic acid combination, ampicillin, cephalothin, cephradine, cloxacillin, enrofloxacin, erythromycin, lincomycin, nafcillin, neomycin, oxacillin, oxytetracycline, penicillin, streptomycin and tetracycline. The susceptibility of the isolates and mean zones of inhibition for each antimicrobial/pathogen combination were assessed.

RESULTS: The proportion of susceptible isolates differed among the species. All isolates were susceptible to the amoxicillin/clavulanic acid combination. Resistance to lincomycin was most frequent (a susceptibility of 8.6%) across all species. Non-susceptible (*i.e.* resistant or intermediate) isolates of *Staph. aureus* were identified from New Zealand and the USA, respectively for all three non-isoxazolyl penicillins (20.6% and 36.0% for amoxicillin, ampicillin and penicillin), and lincomycin (99.9% and 94.6%). Resistance to erythromycin (5.3%) and tetracyclines (6.7%) was detected only in isolates from the USA. There were differences between the susceptibility of *Strep. uberis* and *Strep. dysgalactiae*. All streptococcal isolates demonstrated resistance to aminoglycosides (neomycin 52.4% and streptomycin 27.9%) and enrofloxacin (28%). A resistance of *Strep. dysgalactiae* isolates to tetracycline (almost 100.0%) and oxytetracycline (89.9%) was observed.

CONCLUSION: Most of the mastitis-causing organisms tested were, with the exception of lincosamides, susceptible to most antimicrobials in common use for treatment of bovine

mastitis in both New Zealand and the USA. These results confirm the value of national surveys to test the susceptibility of mastitis causing organisms.

CLINICAL RELEVANCE: This study provides data on the antimicrobial susceptibility patterns of three common mastitis causing organisms (*Staph. aureus*, *Strep. uberis* and *Strep. dysgalactiae*) in New Zealand and the USA.

KEY WORDS: Mastitis, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, susceptibility, antimicrobial.

CLSI - Clinical and Laboratory Standards Institute; MIC - Minimal Inhibitory concentrations; PBP - Penicillin Binding Protein

5.2 Introduction

Changes in susceptibility to antimicrobials of mastitis-causing organisms could result in changes in treatment efficacy. Antimicrobial susceptibility testing of mastitis-causing organisms can be carried out using different methodologies (Constable and Morin 2003). One of the oldest standardised methodologies is the disk diffusion test (Bauer *et al.* 1966). This method is routinely used for susceptibility testing in many countries, including New Zealand and the USA. However, with a few exceptions such as pirlimycin and the combination of penicillin/novobiocin (Thornsberry *et al.* 1993; Thornsberry *et al.* 1997), breakpoints (size of zone of inhibition at which isolates are categorised as susceptible, intermediate or resistant) for interpretation of antimicrobial susceptibility (Watts *et al.* 2008) have not been validated for mastitis-causing organisms. Therefore, for most antimicrobial-pathogen combinations, commercial laboratories use breakpoints of growth inhibition zones that have been established for other pathogens. Furthermore, there are several antimicrobials which are widely used for the treatment of mastitis that are not routinely incorporated in susceptibility testing. Instead, class representative antimicrobials are used for testing, with the assumption that these adequately represent the other antimicrobials of the group in susceptibility tests. For example, a widespread assumption between veterinary practitioners is that bacteria belonging to the Genus *Streptococcus* display homogeneous susceptibility to beta-lactams.

Mastitis is the main reason for antimicrobial use in dairy cattle. Hence, the extensive use of antimicrobials for treatment and prophylaxis of mastitis could result in decreased

susceptibility among mastitis-causing organisms and other pathogens of importance to animal and human health unless the susceptibility of the mastitis-causing organisms is known and behaviour of use of antimicrobials that develop resistance is changed. This creates a problem for clinicians, because antimicrobial treatment generally has to be initiated before the results of culturing and susceptibility tests are available. The initial choice of antimicrobial is often based on population-based historical information on the susceptibility of likely causal agents. The choice of antimicrobial compounds can be guided by the known susceptibility patterns of a range of bacterial isolates from bovine milk. Furthermore, analysis of changes in susceptibility to antimicrobial compounds is essential to guide the choice of appropriate treatments of bovine mastitis and to monitor whether antimicrobial resistance is developing. Local information is essential because significant differences in aetiology, incidence, management and treatment products may exist between regions and countries.

Comparing the results obtained by disk-diffusion test between two countries may indicate existing or emerging problems. However, using rigid breakpoints and reporting only differences in the proportion of susceptible organisms between countries may be insufficient to reveal differences in trends. The size of the zones of inhibition obtained in the disk-diffusion test is usually correlated with the minimal inhibitory concentrations (MIC) and the treatment outcome (Gerber and Craig 1981; Thornsberry *et al.* 1982). Differences in the size of zones of inhibition for tested pathogens between countries may be clinically important since they are likely to be related to differences in the required dose of antimicrobial to achieve a favourable treatment outcome.

This study assessed and compared the susceptibility of common mastitis-causing organisms isolated from milk samples collected in New Zealand or the USA to selected antimicrobials using the disk diffusion test. Differences in the size of zones of inhibition for particular mastitis-causing organism/antimicrobial combinations between the two countries were also estimated. In addition, the assumption that testing a class representative or susceptibility at a genus level is sufficient for the two species mastitis-causing streptococci was challenged.

5.3 Materials and methods

Bacterial isolates from bovine milk samples collected in New Zealand were provided by five commercial Veterinary Diagnostic Laboratories in Auckland, Christchurch, Dunedin,

Hamilton and Palmerston North. Samples were collected during 2006 and 2007. Information provided by the submitting laboratories on each isolate included the microbial species, the abundance of its growth and the laboratory and region of origin of the milk sample. Isolates from the USA (Illinois, Iowa, Kansas, Michigan, Minnesota, Missouri, North Dakota, South Dakota, Virginia and Wisconsin) were collected during 2007 and were obtained from the Laboratory for Udder Health at the University of Minnesota, Veterinary Diagnostic Laboratory, Saint Paul. Accompanying information included the microbial species and the state of origin. To prevent over-representation at the farm level, all tested isolates were from different farms of origin. Isolates for a farm were chosen at random.

The isolates were submitted on Dorset egg-slopes (*Fort Richard Laboratories Ltd*, Auckland, New Zealand). At the *Microbiology Laboratory* of the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University, isolates were re-cultured on 5% sheep blood agar plates (*Fort Richard Laboratories Ltd*), re-identified, suspended in 15% glycerol broth and frozen at -80°C for future reference. Before antibacterial susceptibility testing (Figures 5.2 and 5.3), isolates were again re-cultured on 5% sheep blood agar plates. Thereafter, disk diffusion testing was carried out using the methods recommended by the Clinical and Laboratory Standards Institute (CLSI), as specified in guideline M31-A3 (Watts *et al.* 2008). Antimicrobials that were used for testing susceptibility are listed in Table 5.1. For quality assurance, on each day when disc diffusion plates were being seeded, an appropriate reference strain (*Staph. aureus* ATCC25923 or *Strep. pneumoniae* ATCC49619; *Oxoid*, Auckland, New Zealand) was included. Since the CLSI guideline does not provide interpretive criteria for many of the mastitis pathogen/antimicrobial combinations, whenever guidelines for testing and breakpoints were not available for a specific pathogen, guidelines for other pathogen/antimicrobial combinations of the same group were used. Such a method is commonly used by commercial laboratories and has been reported in the literature (Gentilini *et al.* 2002; Makovec and Ruegg 2003; Pitkala *et al.* 2004). Thus, the breakpoints for ampicillin were used for amoxicillin, those for oxacillin were used for cloxacillin and nafcillin, those for cephalothin were used for cephradine, those for clindamycin were used for lincomycin and those for tetracycline were used for oxytetracycline. Lincomycin disks were used for testing susceptibility to lincosamides as a product containing this antimicrobial is available in the market in New Zealand.

Moreover, the CLSI M31-A3 guideline lacks breakpoints for streptomycin and neomycin. Thus, for these two antimicrobials, the breakpoints originally established by Bauer *et al.* (1966) were used. The breakpoints used are presented in Tables 5.2 and 5.3.

5.3.1 Statistical analysis

The binary outcome variable was the susceptibility testing result (resistant=0 or susceptible=1) for a particular mastitis-causing organism/antimicrobial combination. All isolates with intermediate susceptibility were re-classified as resistant. The proportion of susceptible isolates of a particular organism to a particular antimicrobial was the outcome of interest. The data were analysed in a logistic regression model accounting for the antimicrobial, country of origin and their interaction. The geometric means of susceptibility and their standard errors were obtained and back-transformed to the binomial scale. Analyses were undertaken using SAS (Statistical Analysis System, *SAS Institute Inc.*, Cary, NC, USA 2003) version 9.2., using the GLIMMIX procedure.

Table 5.1. Disk potency of antimicrobials used in the study (μg - micrograms)

Antimicrobial group	Subgroup	Antimicrobial	Disk potency (μg)
Beta-lactam	<i>Non-isoxazolyl penicillin</i>	Amoxicillin	25
		Ampicillin	10
		Penicillin	10
	<i>Non-isoxazolyl penicillin and beta-lactamase inhibitor</i>	Amoxicillin/clavulanic acid	30
		Cloxacillin	5
	<i>Isoxazolyl penicillin</i>	Nafcillin	5
		Oxacillin	1
		<i>Cephalosporin</i>	Cephalothin
	Cephradine		30
	Macrolides /lincosamides	<i>Macrolide</i>	Erythromycin
<i>Lincosamide</i>		Lincomycin	2
Aminoglycosides		Neomycin	30
		Streptomycin	10
Tetracyclines		Oxytetracycline	30
		Tetracycline	30
Quinolones		Enrofloxacin	5

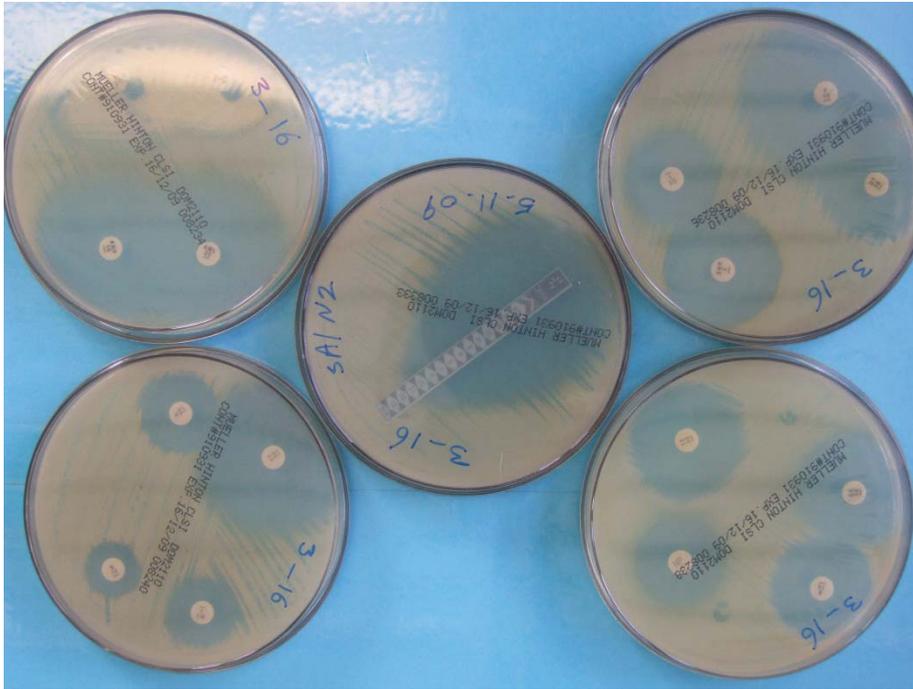


Figure 5.1 Agar disk diffusion and E-test of *Staphylococcus* isolate



Figure 5.2 Agar disk diffusion and E-test of streptococcal isolate

The susceptibility of a mastitis-causing organism to an antimicrobial was classified as being 'high' when the overall susceptibility to an antimicrobial was $\geq 90.0\%$, 'moderate' when it was between 75.0-89.9%, 'low' when it was 50.0-74.9% and 'resistant' when it was $< 50.0\%$.

Data for the diameters of the zones of inhibition for susceptible and resistant (including 'intermediate') isolates were subjected to analysis of variance with respect to country of origin and mastitis-causing organism/antimicrobial combinations (using the MIXED procedure of SAS).

The level of agreement of the number of observations of the binary outcome (susceptible/resistant isolate) for a specific mastitis-causing organism (*i.e. Staph. aureus, Strep. uberis, Strep. dysgalactiae*) and related antimicrobials (*i.e. macrolides and lincosamides, members of beta-lactams such as penicillin and oxacillin*) was tested using the KAPPA function of the FREQ procedure of SAS measuring the chance-corrected measure of agreement.

The level of significance was set at $P < 0.05$.

5.4 Results

There were 107, 106, and 41 isolates of *Staph. aureus*, *Strep. uberis*, and *Strep. dysgalactiae*, from New Zealand and 75, 20, and 48 respectively from the USA (Tables 5.2 and 5.3). Antimicrobial disk diffusion tests were carried out on 6262 organism/ antimicrobial combinations (Table 5.2).

Table 5.2. Susceptibility of isolates of *Staphylococcus aureus* and streptococci by country

Antimicrobial group	Interp ratio n	Zone diameter breakpoints			<i>Staphylococcus aureus</i>						Zone diameter breakpoints						<i>Streptococcus</i> species							
		Zone diameter breakpoints			New Zealand			USA			Total			New Zealand			USA			Total				
		n ¹	% ²	n	n ¹	%	n	n ¹	%	n	n ¹	%	n	n ¹	%	n	n ¹	%	n	n ¹	%	n	n ¹	%
Antimicrobial	S ³	>28	79.4	48	64.0	133	73.1	>25	142	96.6	67	98.5	209	97.2										
	I ⁴	NA ⁶	NA	NA	NA	NA	NA	19-25	5	4.9	1	1.5	6	2.8										
	R ⁵	<29	20.6	27	36.0	49	26.9	<19	0	0.0	0	0.0	0	0.0										
Amoxicillin / Clavulanic acid	S	>19	100.0	75	100.0	182	100.0	>17	147	100.0	68	100.0	215	100.0										
	I	NA	NA	NA	NA	NA	NA	14-17	0	0.0	0	0.0	0	0.0										
	R	<20	0.0	0	0.0	0	0.0	<14	0	0.0	0	0.0	0	0.0										
Ampicillin	S	>28	79.4	48	64.0	133	73.1	>25	133	90.5	66	97.1	199	92.6										
	I	NA	NA	NA	NA	NA	NA	19-25	14	13.4	2	2.9	16	7.4										
	R	<29	20.6	27	36.0	49	26.9	<19	0	0.0	0	0.0	0	0.0										
Beta-lactams	S	>12	100.0	75	100.0	182	100.0	>12	146	99.3	68	100.0	214	99.5										
	I	11-12	0.0	0	0.0	0	0.0	11-12	0	0.0	0	0.0	0	0.0										
	R	<11	0.0	0	0.0	0	0.0	<11	1	1.0	0	0.0	1	0.5										
Cloxacillin	S	>12	100.0	75	100.0	182	100.0	>12	76	96.2	55	80.9	131	89.1										
	I	11-12	0.0	0	0.0	0	0.0	11-12	3	3.0	5	7.4	8	5.4										
	R	<11	0.0	0	0.0	0	0.0	<11	0	0.0	8	11.8	8	5.4										
Nafcillin	S	>12	100.0	75	100.0	182	100.0	>12	145	98.6	56	82.4	201	93.5										
	I	11-12	0.0	0	0.0	0	0.0	11-12	1	1.0	0	0.0	1	0.5										
	R	<11	0.0	0	0.0	0	0.0	<11	1	1.0	12	17.7	13	6.1										
Oxacillin	S	>28	79.4	48	64.0	133	73.1	>23	146	99.3	68	100.0	214	99.5										
	I	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA										
	R	<29	20.6	27	36.0	49	26.9	<24	1	1.0	0	0.0	1	0.5										

Table 5.2. Susceptibility of isolates *Staphylococcus aureus* and streptococci by country – Continued

	S	>17	107	100.0	75	100.0	182	100.0	>17	146	99.3	67	98.5	213	99.1
Cephalothin	I	15-17	0	0.0	0	0.0	0	0.0	15-17	0	0.0	0	0.0	0	0.0
	R	<15	0	0.0	0	0.0	0	0.0	<15	1	1.0	1	1.5	2	0.9
	S	>17	85	100.0	75	100.0	182	100.0	>17	146	99.3	67	98.5	213	99.1
Cephadrine	I	15-17	0	0.0	0	0.0	0	0.0	15-17	0	0.0	0	0.0	0	0.0
	R	<15	0	0.0	0	0.0	0	0.0	<15	1	1.0	1	1.5	2	0.9
	S	>22	107	100.0	71	94.7	178	97.8	>20	144	98.0	67	98.5	211	98.1
Erythromycin	I	14-22	0	0.0	4	5.3	4	2.2	16-20	1	1.0	0	0.0	1	0.5
	R	<14	0	0.0	0	0.0	0	0.0	<16	2	2.0	1	1.5	3	1.4
Macrolides /lincosamides	S	>20	1	0.9	4	5.3	5	2.8	>20	42	28.6	2	2.9	44	20.5
Lincomycin	I	15-20	106	99.1	66	88	172	94.5	15-20	103	77.1	41	60.3	144	67.0
	R	<15	0	0.0	5	6.7	5	2.8	<15	2	1.9	25	36.8	27	12.6
	S	>14	107	100.0	75	100.0	182	100.0	>14	0	0.0	1	1.5	1	0.5
Neomycin	I	13-14	0	0.0	0	0.0	0	0.0	13-14	1	0.7	1	1.5	2	0.9
	R	<13	0	0.0	0	0.0	0	0.0	<13	146	99.5	66	97.1	212	98.6
Amnoglycosides	S	>9	106	99.1	74	98.7	180	98.9	>9	30	20.4	48	70.6	78	36.3
Streptomycin	I	7-9	0	0.0	0	0.0	0	0.0	7-9	12	8.2	0	0.0	12	5.6
	R	<7	1	0.9	1	1.3	2	1.1	<7	105	71.4	20	29.4	125	58.1
	S	>18	107	100.0	70	93.3	177	97.3	>22	110	74.8	10	14.7	120	55.8
Oxytetracycline	I	15-18	0	0.0	0	0.0	0	0.0	19-22	37	33.1	22	32.4	59	27.4
	R	<15	0	0.0	5	6.7	5	2.8	<19	0	0.0	36	52.9	36	16.7
Tetracyclines	S	>18	107	100.0	70	93.3	177	97.3	>22	102	69.4	6	8.8	108	50.2
Tetracycline	I	15-18	0	0.0	0	0.0	0	0.0	19-22	44	38.5	21	30.9	65	30.2
	R	<15	0	0.0	5	6.7	5	2.8	<19	1	0.9	41	60.3	42	19.5
	S	>22	107	100.0	75	100.0	182	100.0	>22	79	53.7	26	38.2	105	48.8
Quinolones	I	17-22	0	0.0	0	0.0	0	0.0	17-22	68	55.9	42	61.8	110	51.2
	R	<17	0	0.0	0	0.0	0	0.0	<17	0	0.0	0	0.0	0	0.0

¹number of tested isolates; ²percent of tested isolates; ³susceptible; ⁴intermediate; ⁵resistant; ⁶not applicable

Table 5.3. Susceptibility of streptococcal isolates by country

Antimicrobial group	Antimicrobial	Interpretation	Zone diameter breakpoints	<i>Streptococcus uberis</i>				<i>Streptococcus dysgalactiae</i>								
				New Zealand		USA		New Zealand		USA		Total				
				n ¹	% ²	n	%	n	%	n	%	n	%	n	%	
Beta-lactams	Amoxicillin	S ³	>25	101	95.3	19	95.0	120	100.0	41	100.0	48	100.0	89	100.0	
		I ⁴	19-25	5	4.7	1	5.0	6	4.8	0	0.0	0	0.0	0	0.0	
		R ⁵	<19	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	
	Amoxicillin / Clavulanic acid	S	>17	106	100.0	20	100.0	126	100.0	41	100.0	48	100.0	89	100.0	
		I	14-17	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	
	Ampicillin	R	<14	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	
		S	>25	94	88.7	18	90.0	112	88.9	39	95.1	48	100.0	87	100.0	
		I	19-25	12	11.3	2	10.0	14	11.1	2	4.9	0	0.0	0	0.0	
	Beta-lactams	Cloxacillin	R	<19	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
			S	>12	105	99.1	20	100.0	125	99.2	41	100.0	48	100.0	89	100.0
I			11-12	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	
Nafcillin		R	<11	1	0.9	0	0.0	1	0.8	0	0.0	0	0.0	0	0.0	
		S	>12	76	96.2	7	35.0	83	83.8	NT ⁶	NT	48	100.0	48	100.0	
		I	11-12	3	3.8	5	25.0	8	8.1	NT	NT	0	0.0	0	0.0	
Oxacillin		R	<11	0	0.0	8	40.0	8	8.1	NT	NT	0	0.0	0	0.0	
		S	>12	104	98.1	8	40.0	112	88.9	41	100.0	48	100.0	89	100.0	
		I	11-12	1	0.9	0	0.0	1	0.8	0	0.0	0	0.0	0	0.0	
Penicillin		R	<11	1	0.9	12	60.0	13	10.3	0	0.0	0	0.0	0	0.0	
	S	>23	105	99.1	20	100.0	125	99.2	41	100.0	48	100.0	89	100.0		
	I	NA ⁷	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		
	R	<24	1	0.9	0	0.0	1	0.8	0	0.0	0	0.0	0	0.0		

Table 5.3. Susceptibility of streptococcal isolates by country - Continued

Cephalothin	S	>17	105	99.1	19	95.0	124	98.4	41	100.0	48	100.0	89	100.0
	I	15-17	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	R	<15	1	0.9	1	5.0	2	1.6	0	0.0	0	0.0	0	0.0
Cephradine	S	>17	105	99.1	19	95.0	124	98.4	41	100.0	48	100.0	89	100.0
	I	15-17	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
	R	<15	1	0.9	1	5.0	2	1.6	0	0.0	0	0.0	0	0.0
Erythromycin	S	>20	103	97.2	19	95.0	122	96.8	41	100.0	48	100.0	89	100.0
	I	16-20	1	0.9	0	0.0	1	0.8	0	0.0	0	0.0	0	0.0
	R	<16	2	1.9	1	5.0	3	2.4	0	0.0	0	0.0	0	0.0
Macrolides /lincosamides	S	>20	42	39.6	2	10.0	44	34.9	0	0.0	0	0.0	0	0.0
	I	15-20	62	58.5	2	10.0	64	50.8	41	100.0	39	81.3	80	89.9
	R	<15	2	1.9	16	80.0	18	14.3	0	0.0	9	18.8	9	10.1
Neomycin	S	>14	0	0.0	1	5.0	1	0.8	0	0.0	0	0.0	0	0.0
	I	13-14	1	0.9	0	0.0	1	0.8	0	0.0	1	2.1	1	1.1
	R	<13	105	99.1	19	95.0	124	98.4	41	100.0	47	97.9	88	98.9
Aminoglycosides	S	>9	1	0.9	1	5.0	2	1.6	29	70.7	47	97.9	76	85.4
	I	7-9	0	0.0	0	0.0	0	0.0	0	0.0	12	29.3	12	8.8
	R	<7	105	99.1	19	95.0	124	98.4	0	0.0	1	2.1	1	1.1
Oxytetracycline	S	>22	105	99.1	6	30.0	111	88.1	5	12.2	4	8.3	9	10.1
	I	19-22	1	0.9	2	10.0	3	2.4	36	87.8	20	41.7	56	62.9
	R	<19	0	0.0	12	60.0	12	9.5	0	0.0	24	50.0	24	27.0
Tetracyclines	S	>22	101	95.3	6	30.0	107	84.9	1	2.4	0	0.0	1	1.1
	I	19-22	4	3.8	0	0.0	4	3.2	40	97.6	21	43.8	61	68.5
	R	<19	1	0.9	14	70.0	15	11.9	0	0.0	27	56.3	27	30.3
Quinolones	S	>22	66	62.3	10	50.0	76	60.3	66	13	31.7	16	33.3	29
	I	17-22	40	37.7	10	50.0	50	39.7	28	68.3	32	67.7	60	67.4
	R	<17	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0

¹number of tested isolates; ²percent of tested isolates; ³susceptible; ⁴intermediate; ⁵resistant; ⁶Not tested; ⁷Not applicable

5.4.1 Level of susceptibility

Microbial susceptibility

All isolates were susceptible to an amoxicillin/clavulanic acid combination. Additionally, there was a high susceptibility to cloxacillin (almost 100.0%), nafcillin (96.1%), oxacillin (97.4%), cephalosporins (cephalothin and cephradine ~ 99.5%) and erythromycin (98.0%). The susceptibility was moderate to non-isoxazolyl penicillins (amoxicillin 85.5%; ampicillin 83.0%; and penicillin 86.8%), low to enrofloxacin (72.0%), streptomycin (72.1%) and tetracyclines (oxytetracycline ~ 73.2% and tetracycline ~ 69.7%). Resistance was detected to lincomycin (susceptibility of 8.6%) and neomycin (susceptibility of 47.6%) as shown in Table 5.4.

The susceptibility of causative organisms was higher in New Zealand than in USA isolates to amoxicillin (89.4% *vs.* 80.4%; $P=0.015$), lincomycin (16.9% *vs.* 4.2%; $P<0.001$), nafcillin (98.4% *vs.* 90.9%; $P=0.006$), oxacillin (99.2% *vs.* 91.6%; $P=0.002$), penicillin (90.9% *vs.* 81.1%; $P=0.006$), and tetracyclines (oxytetracycline: 85.4% *vs.* 55.9%; $P<0.001$; tetracycline: 82.3% *vs.* 53.2%; $P<0.001$). Conversely, the susceptibility in isolates from New Zealand was lower than in those from the USA for aminoglycosides (neomycin: 42.1% *vs.* 53.2%; $P=0.035$; and streptomycin: 53.5% *vs.* 85.3%; $P<0.001$). There were differences between countries in the susceptibilities to the other antimicrobials ($P>0.05$; Table 5.4).

The aggregated susceptibilities of all isolates to all antimicrobials and the corresponding Standard Errors (SE) per country were similar (isolates from New Zealand: $81.2\pm 0.51\%$, isolates from the USA: $74.4\pm 0.67\%$; $P=0.084$).

Table 5.4. Prevalence of susceptibility (mean \pm SE) of *Staphylococcus aureus* and streptococci isolated from milk samples collected in New Zealand and the USA to a range of antimicrobials

Antibiotic group	Antibiotic	<i>Staphylococcus aureus</i>			<i>Streptococcus</i> species			All isolates		
		NZ ¹	USA ²	Total	NZ	USA	Total	NZ	USA	Total
Beta-lactams	Amoxicillin	79.4 \pm 4.18	64.0 \pm 5.95*	72.4 \pm 3.86	96.6 \pm 1.51	98.5 \pm 1.45	97.8 \pm 1.19	89.4 \pm 2.16	80.4 \pm 3.72*	85.5 \pm 2.23
	Amoxicillin / Clavulanic acid	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	Ampicillin	79.4 \pm 4.18	64.0 \pm 5.95*	72.4 \pm 3.86	90.5 \pm 2.63	97.01 \pm 2.05	94.7 \pm 1.20	85.8 \pm 2.53	79.7 \pm 3.79	83.0 \pm 2.45
	Cloxacillin	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	99.3 \pm 0.68	100.0 \pm 0.10	100.0 \pm 0.50	99.6 \pm 0.40	100.0 \pm 0.00	100.0 \pm 0.26
	Nafticillin	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	96.2 \pm 2.17	80.9 \pm 5.18*	91.2 \pm 2.80	98.4 \pm 0.91	90.9 \pm 2.54*	96.1 \pm 1.23
	Oxacillin	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	98.6 \pm 0.93	82.4 \pm 4.99*	94.8 \pm 1.89	99.2 \pm 0.54	91.6 \pm 2.44*	97.4 \pm 0.96
Macrolides/Lincosamides	Penicillin	79.4 \pm 4.05	64.0 \pm 5.78*	72.4 \pm 3.66	99.3 \pm 0.68	100.0 \pm 0.00	100.0 \pm 0.50	90.9 \pm 1.97	81.1 \pm 3.66*	86.8 \pm 2.11
	Cephalothin	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	99.3 \pm 0.66	98.5 \pm 1.45	99.0 \pm 0.67	99.6 \pm 0.38	99.3 \pm 0.69	99.5 \pm 0.35
	Cephazidine	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	99.3 \pm 0.66	98.5 \pm 1.45	99.0 \pm 0.67	99.6 \pm 0.42	99.3 \pm 0.69	99.5 \pm 0.37
	Erythromycin	100.0 \pm 0.00	94.7 \pm 2.61	100.0 \pm 1.15	98.0 \pm 1.15	98.5 \pm 1.45	98.3 \pm 0.97	98.8 \pm 0.66	96.5 \pm 1.55	98.0 \pm 0.72
	Lincomycin	0.9 \pm 0.92	5.3 \pm 2.61	2.3 \pm 1.22	28.6 \pm 4.62	2.9 \pm 2.05*	9.9 \pm 3.42	16.9 \pm 2.79	4.2 \pm 1.70*	8.6 \pm 1.91
	Neomycin	100.0 \pm 0.00	100.0 \pm 0.00	100.00 \pm 0.00	0.0 \pm 0.00	1.5 \pm 1.45	0.0 \pm 0.50	42.1 \pm 4.10	53.2 \pm 5.00*	47.6 \pm 3.80
Aminoglycosides	Streptomycin	99.1 \pm 0.92	98.7 \pm 1.32	98.9 \pm 0.77	20.4 \pm 3.95	70.6 \pm 6.12*	44.0 \pm 5.27	53.5 \pm 4.17	85.3 \pm 3.24*	72.1 \pm 3.45
Tetracyclines	Oxytetracycline	100.0 \pm 0.00	93.3 \pm 2.91	100.0 \pm 1.29	74.8 \pm 4.37	14.7 \pm 4.58*	41.7 \pm 5.72	85.4 \pm 2.57	55.9 \pm 4.97*	73.2 \pm 3.20
	Tetracycline	100.0 \pm 0.00	93.3 \pm 2.91	100.0 \pm 1.29	69.4 \pm 4.75	8.8 \pm 3.56*	31.9 \pm 5.73	82.3 \pm 2.85	53.2 \pm 5.00*	69.7 \pm 3.38
Quinolones	Enrofloxacin	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	53.7 \pm 5.32	38.2 \pm 6.71*	45.9 \pm 5.02	73.2 \pm 3.51	70.6 \pm 4.43	72.0 \pm 3.22

¹New Zealand; ²The United States of America; * different from the prevalence of susceptibility in New Zealand for the same antimicrobial/causative organism combination (P<0.05)

Susceptibility of *Staphylococcus aureus*

All isolates of *Staph. aureus* were susceptible to the combination of amoxicillin/clavulanic acid, the isoxazolyl penicillins (cloxacillin, nafcillin and oxacillin), cephalosporins (cephalothin and cephradine), neomycin and enrofloxacin. Additionally, there was a high susceptibility to erythromycin (almost 100%), streptomycin (98.9%) and tetracyclines (almost 100%). The susceptibility of *Staph. aureus* isolates was low to non-isoxazolyl penicillins (amoxicillin, ampicillin and penicillin 72.4%) and they were resistant to lincomycin (susceptibility of 2.3%; Table 5.4).

The susceptibility of isolates of *Staph. aureus* to non-isoxazolyl penicillins from New Zealand (79.4%) was higher than in those from the USA (64.0%; $P=0.02$). Differences between the susceptibility of *Staph. aureus* isolates to other antimicrobials between the two countries were non-significant ($P>0.05$; Table 5.4).

Susceptibility of *Streptococcus* spp

All *Streptococcus* isolates were susceptible to the amoxicillin/clavulanic acid combination. Additionally, susceptibility to cephalosporins was 99.0%, erythromycin 98.3%, isoxazolyl penicillins (cloxacillin: almost 100.0%, nafcillin: 91.2% and cloxacillin: 94.8%) and non-isoxazolyl penicillins (amoxicillin: 97.8%; ampicillin: 94.7%; and penicillin: almost 100.0%). Resistance of streptococci was detected to aminoglycosides (susceptibility of almost 0.0% for neomycin and 44.0% for streptomycin), lincomycin (9.9%), enrofloxacin (46.0%) and tetracyclines (oxytetracycline: 41.7% and tetracycline: 31.9%).

More *Streptococcus* isolates from New Zealand were susceptible to enrofloxacin (53.7% vs. 38.2%; $P=0.036$), lincomycin (28.6% vs. 2.9%; $P<0.001$), nafcillin (96.2% vs. 80.9%; $P=0.007$), oxacillin (98.6% vs. 82.4%; $P<0.001$) and tetracyclines (oxytetracycline 74.8% vs. 14.7%; $P<0.001$; and tetracycline 69.4% vs. 8.8%; $P<0.001$), but susceptibility to streptomycin (20.4% vs. 70.6%; $P<0.001$) was lower. The differences in susceptibilities to other antimicrobials between the two countries were non-significant ($P>0.05$).

***Streptococcus uberis*.** All isolates of *Strep. uberis* were susceptible to an amoxicillin/clavulanic acid combination (Table 5.5). Additionally, there was a high susceptibility to amoxicillin (95.1%), cloxacillin (almost 100.0%), cephalosporins

(97.8%), erythromycin (96.2%) and penicillin (almost 100.0%). The susceptibility of *Strep. uberis* isolates was moderate to ampicillin (89.4%), nafcillin (78.7%), oxacillin (85.5%) and oxytetracycline (87.0). The susceptibility to enrofloxacin (56.2%) and tetracycline (74.6%) was low. Resistance of *Strep. uberis* isolates was detected to lincomycin (susceptibility of 21.3%) and aminoglycosides (neomycin susceptibility of 0.0%; and streptomycin susceptibility of 2.2%).

The susceptibility of *Strep. uberis* isolates from New Zealand was higher than in those from the USA for lincomycin (39.6% vs. 10.0%; $P=0.02$), nafcillin (96.2% vs. 35.0%; $P<0.001$), oxacillin (98.1% vs. 40.0%; $P<0.001$) and tetracyclines (oxytetracycline: 99.1% vs. 30.0; $P<0.001$; and tetracycline: 95.3% vs. 30.0%; $P<0.001$). The differences in the susceptibilities to other antimicrobials between the two countries were non-significant ($P>0.05$; Table 5.5).

Streptococcus dysgalactiae. Isolates of *Strep. dysgalactiae* from New Zealand were not tested for susceptibility to nafcillin. Therefore, nafcillin was excluded from the analysis for this organism. All isolates of *Strep. dysgalactiae* were susceptible to most beta-lactam antimicrobials (amoxicillin, amoxicillin/clavulanic acid combination, cephalothin, cephadrine cloxacillin, oxacillin, penicillin) and erythromycin. There was a high susceptibility to ampicillin (almost 100%) and streptomycin (91.4%) and a resistance to enrofloxacin (susceptibility of 32.5%), lincomycin (susceptibility of 0.0%), neomycin (susceptibility of 0.0%) and tetracyclines (oxytetracycline susceptibility of 10.1%; and tetracycline susceptibility of 0.0%; Table 5.5).

The susceptibility of *Strep. dysgalactiae* isolates from New Zealand was lower than in those from the USA for streptomycin (70.7% vs. 97.9%; $P=0.005$). There were no differences in the susceptibility of *Strep. dysgalactiae* isolates from New Zealand and the USA to other antimicrobials ($P>0.05$; Table 5.5).

Table 5.5. Prevalence of susceptibility (mean \pm SE) of *Streptococcus uberis* and *Strep. dysgalactiae* isolated from milk samples collected in New Zealand and the USA to a range of antimicrobials

Antibiotic group	Antibiotic	<i>Streptococcus uberis</i>			<i>Streptococcus dysgalactiae</i>		
		NZ ¹	USA ²	Total	NZ	USA	Total
Beta-lactams	Amoxicillin	95.3 \pm 2.07	95.0 \pm 4.88	95.1 \pm 2.61	100.0 \pm 0.01	100.0 \pm 0.01	100.0 \pm 0.01
	Amoxicillin / Clavulanic acid	100.0 \pm 0.00	100.0 \pm 0.01	100.0 \pm 0.01	100.0 \pm 0.01	100.0 \pm 0.00	100.0 \pm 0.01
	Ampicillin	88.7 \pm 3.19	90.0 \pm 6.75	89.4 \pm 3.91	95.1 \pm 3.37	100.0 \pm 0.00	100.0 \pm 1.63 [#]
	Cloxacillin	99.1 \pm 0.94	100.0 \pm 0.01	100.0 \pm 1.09	100.0 \pm 0.01	100.0 \pm 0.01	100.0 \pm 0.01
	Nafcillin	96.2 \pm 2.16	35.0 \pm 10.87 [*]	78.7 \pm 6.42	NE ³	100.0 \pm 0.00	NE
Macrolides/ Lincosamides	Oxacillin	98.1 \pm 1.31	40.0 \pm 11.18 [*]	85.5 \pm 5.27	100.0 \pm 0.01	100.0 \pm 0.01	100.0 \pm 0.01
	Penicillin	99.1 \pm 0.94	100.0 \pm 0.01	100.0 \pm 1.09	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	Cephalothin	99.1 \pm 0.93	95.0 \pm 4.88	97.8 \pm 1.52	100.0 \pm 0.01	100.0 \pm 0.01	100.0 \pm 0.01
	Cephradine	99.1 \pm 0.93	95.0 \pm 4.88	97.8 \pm 1.52	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	Erythromycin	97.2 \pm 1.61	95.0 \pm 4.88	96.2 \pm 2.15	100.0 \pm 0.01	100.0 \pm 0.01	100.0 \pm 0.01
Aminoglycosides	Lincomycin	39.6 \pm 5.25	10.0 \pm 6.75 [*]	21.3 \pm 6.62	0.0 \pm 0.01	0.0 \pm 0.16	0.0 \pm 0.00
	Neomycin	0.0 \pm 0.00	5.0 \pm 4.88	0.0 \pm 1.11	0.0 \pm 0.00	0.0 \pm 0.00	0.0 \pm 0.01
Tetracyclines	Streptomycin	0.9 \pm 0.93	5.0 \pm 4.88	2.2 \pm 1.52	70.7 \pm 7.62	97.9 \pm 2.07 [*]	91.4 \pm 4.26 [#]
	Oxytetracycline	99.1 \pm 0.93	30.0 \pm 10.43 [*]	87.0 \pm 6.23	12.2 \pm 5.22	8.3 \pm 4.05	10.1 \pm 3.33 [#]
	Tetracycline	95.3 \pm 2.07	30.0 \pm 10.43 [*]	74.6 \pm 6.50	2.4 \pm 2.41	0.0 \pm 0.01	0.0 \pm 1.14 [#]
Quinolones	Enrofloxacin	62.3 \pm 5.20	50.0 \pm 11.42	56.2 \pm 6.46	31.7 \pm 7.35	33.3 \pm 7.09	32.5 \pm 5.39 [#]

¹New Zealand; ²The United States of America; ³Not estimated; * different from the prevalence of susceptibility in New Zealand for the same antimicrobial/causative organism combination (P<0.05); # different from the prevalence of susceptibility of *Streptococcus uberis*

5.4.2 Zones of inhibition

The diameters of the zones of inhibition were evaluated independently for the isolates that tested susceptible and those that tested resistant (including ‘intermediate’). They were affected by the country of origin of the isolate. The diameters of zones of inhibition, when different ($P < 0.05$), were smaller for the isolates of *Staph. aureus* from New Zealand, for most antimicrobials for susceptible and resistant isolates (Table 5.6), except for lincomycin (both, resistant and susceptible isolates) and to streptomycin (only resistant isolates).

The diameters of zones of inhibition for streptococci from New Zealand were larger for the isolates resistant to nafcillin (11.7 vs. 6.8 cm; $P = 0.008$), lincomycin (18.4 vs. 12.2 cm; $P < 0.001$) and tetracyclines (oxytetracycline 21.2 vs. 16.8 cm; $P < 0.001$; and tetracycline 20.6 vs. 16.0 cm; $P < 0.001$). The diameters of zones of inhibition for streptococci from New Zealand were smaller for the susceptible isolates to nafcillin (18.5 vs. 19.5 cm; $P = 0.04$), and isolates resistant to streptomycin (2.8 vs. 7.7 cm; $P = 0.008$; Table 5.4.2.1). The variability in the size of zones of inhibition for isolates of *Strep. uberis* and *Strep. dysgalactiae* are presented in Table 5.7. The variability in the size of zones of inhibition for isolates of *Strep. uberis* and *Strep. dysgalactiae* was different from that of the *Streptococcus* spp.

Table 5.6. Diameters of zones of inhibition (mean \pm SE) for susceptible and resistant isolates of *Staphylococcus aureus* and streptococci isolated from milk samples collected in New Zealand and the USA

Antimicrobial group	Antimicrobial	Interpretation	<i>Staphylococcus aureus</i>			<i>Streptococcus</i> species			
			New Zealand	USA	Total	New Zealand	USA	Total	
	Amoxicillin	S ¹	34.3 \pm 0.16	37.3 \pm 0.38*	34.8 \pm 0.16	31.1 \pm 0.24	30.9 \pm 0.34	31.1 \pm 0.20	
		R ²	17.3 \pm 0.25	19.4 \pm 0.50*	17.7 \pm 0.25	24.0 \pm 1.26	24.0 \pm 2.82	24 \pm 1.21	
	Amoxicillin / Clavulanic acid	S	32.2 \pm 0.14	33.3 \pm 0.30*	32.4 \pm 0.13	32.4 \pm 0.23	33.0 \pm 0.34	32.6 \pm 0.20	
		R	19.0 \pm 2.62	NA ³	19.0 \pm 2.84	NA	NA	NA	
	Ampicillin	S	34.9 \pm 0.16	37.2 \pm 0.38*	35.2 \pm 0.16	30.1 \pm 0.24	30 \pm 0.35	30.1 \pm 0.21	
		R	16.1 \pm 0.25	18.1 \pm 0.50*	16.5 \pm 0.25	24.2 \pm 0.75	23.5 \pm 1.99	24.1 \pm 0.74	
	Cloxacillin	S	28.1 \pm 0.14	30.8 \pm 0.30*	28.5 \pm 0.13	23.6 \pm 0.23	23.8 \pm 0.34	23.7 \pm 0.20	
		R	NA	NA	NA	0 \pm 2.82	NA	0 \pm 2.96	
	Beta-lactams	Nafcillin	S	20.6 \pm 0.25	21.5 \pm 0.30*	21.0 \pm 0.21	18.5 \pm 0.32	19.5 \pm 0.38*	18.9 \pm 0.26
			R	NA	NA	NA	11.7 \pm 1.63	6.8 \pm 0.78*	7.8 \pm 0.74
Oxacillin		S	21.0 \pm 0.14	23.2 \pm 0.30*	21.3 \pm 0.14	21 \pm 0.23	21.9 \pm 0.38	21.3 \pm 0.21	
		R	11.0 \pm 2.62	NA	11.0 \pm 2.84	6.0 \pm 1.99	5.4 \pm 0.81	5.5 \pm 0.79	
Penicillin		S	36.8 \pm 0.16	40.8 \pm 0.38*	37.4 \pm 0.16	31.9 \pm 0.23	31.6 \pm 0.34	31.8 \pm 0.20	
		R	15.6 \pm 0.26	17.1 \pm 0.50*	15.9 \pm 0.25	13.0 \pm 2.82	7.7 \pm 0.34	13.0 \pm 2.96	
Cephalothin		S	33.2 \pm 0.14	36.0 \pm 0.30*	33.7 \pm 0.13	31.2 \pm 0.23	31.6 \pm 0.34	31.3 \pm 0.20	
		R	15.0 \pm 2.62	NA	15.0 \pm 2.84	0.0 \pm 2.82	0.0 \pm 2.82	0.0 \pm 2.09	
Cephradine		S	24.5 \pm 0.14	26.8 \pm 0.30*	24.9 \pm 0.14	29.0 \pm 0.23	29.3 \pm 0.34	29.1 \pm 0.20	
		R	NA	NA	NA	0.0 \pm 2.82	0.0 \pm 2.82	0.0 \pm 2.09	
Macrolides /lincosamides	Erythromycin	S	24.2 \pm 0.16	25.9 \pm 0.31*	24.6 \pm 0.16	24.7 \pm 0.23	24.4 \pm 0.34	24.6 \pm 0.20	
		R	21.1 \pm 0.24	21.5 \pm 1.31	21.1 \pm 0.26	14.3 \pm 1.63	12.0 \pm 2.82	13.8 \pm 1.48	
	Lincomycin	S	22.0 \pm 2.62	21.5 \pm 1.31	21.6 \pm 1.27	23.2 \pm 0.44	21.5 \pm 1.99	23.2 \pm 0.45	
		R	17.7 \pm 0.14	17.2 \pm 0.31	17.7 \pm 0.14	18.4 \pm 0.28	12.2 \pm 0.35*	16.0 \pm 0.23	

Table 5.6 Diameters of zones of inhibition - continued

Aminoglycosides	Neomycin	S	19.8 ± 0.14	21.1 ± 0.30*	20.0 ± 0.13	NA	33.0 ± 2.82	33.0 ± 2.96
		R	NA	NA	NA	9.0 ± 0.23	9.6 ± 0.34	9.2 ± 0.20
	Streptomycin	S	15.1 ± 0.18	15.6 ± 0.37	15.2 ± 0.17	NA	NA	NA
		R	13.7 ± 0.21	13.2 ± 0.53	13.6 ± 0.21	2.8 ± 0.23	7.7 ± 0.34*	4.4 ± 0.20
	Oxytetracycline	S	23.0 ± 0.14	26.7 ± 0.31*	23.6 ± 0.14	26.1 ± 0.28	26.4 ± 0.89	26.1 ± 0.27
		R	NA	10.4 ± 1.17	10.4 ± 1.27	21.2 ± 0.46	16.8 ± 0.37*	18.5 ± 0.30
Tetracyclines	Tetracycline	S	23.8 ± 0.14	27.9 ± 0.31*	24.4 ± 0.14	26.1 ± 0.28	26.3 ± 1.15	26.1 ± 0.28
		R	NA	10.2 ± 1.17	10.2 ± 1.27	20.6 ± 0.42	16.0 ± 0.36*	17.9 ± 0.29
Quinolones	Enrofloxacin	S	26.9 ± 0.14	28.9 ± 0.30*	27.2 ± 0.13	23.8 ± 0.32	23.7 ± 0.55	23.8 ± 0.29
		R	20.0 ± 2.62	NA	20.0 ± 2.84	21.4 ± 0.34	21.3 ± 0.44	21.4 ± 0.28

¹Susceptible; ²Resistant; ³Not applicable; * different from the mean diameter of zones of inhibition in New Zealand for the same antimicrobial/causative organism combination (P<0.05).

Table 5.7. Diameters of zones of inhibition (mean \pm SE) for susceptible and resistant isolates of *Streptococcus uberis* and *Strep. dysgalactiae* isolated from milk samples collected in New Zealand and the USA

Antimicrobial group	<i>Streptococcus uberis</i>				<i>Streptococcus dysgalactiae</i>			
	Antimicrobial	Interpretation	New Zealand	USA	Total	New Zealand	USA	Total
Amoxicillin	S ¹		31.3 \pm 0.25	29.4 \pm 0.58*	31.0 \pm 0.25	30.8 \pm 0.39	31.5 \pm 0.36	31.2 \pm 0.29
	R ²		24.0 \pm 1.12	24.0 \pm 2.51	24.0 \pm 1.12	NA ³	NA	NA
Amoxicillin / Clavulanic acid	S		32.4 \pm 0.24	28.7 \pm 0.56*	31.8 \pm 0.24	32.4 \pm 0.39	34.8 \pm 0.36*	33.7 \pm 0.29
	R		NA	NA	NA	NA	NA	NA
Ampicillin	S		30.0 \pm 0.26	28.4 \pm 0.59*	29.8 \pm 0.26	30.4 \pm 0.4	30.6 \pm 0.36	30.5 \pm 0.29
	R		24.2 \pm 0.72	23.5 \pm 1.78	24.1 \pm 0.73	24.5 \pm 1.78	NA	24.5 \pm 1.94
Cloxacillin	S		23.1 \pm 0.25	18.4 \pm 0.56*	22.3 \pm 0.24	24.9 \pm 0.39	26.1 \pm 0.36*	25.6 \pm 0.29
	R		0.0 \pm 2.51	NA	0 \pm 2.74	NA	NA	NA
Nafcillin	S		18.5 \pm 0.29	17.4 \pm 0.95	18.4 \pm 0.30	NA	19.9 \pm 0.36	19.9 \pm 0.40
	R		11.7 \pm 1.45	6.8 \pm 0.7*	7.8 \pm 0.68	NA	NA	NA
Oxacillin	S		20.7 \pm 0.25	15.4 \pm 0.89*	20.3 \pm 0.26	22 \pm 0.39	22.9 \pm 0.36	22.5 \pm 0.29
	R		6.0 \pm 1.78	5.4 \pm 0.72	5.5 \pm 0.73	NA	NA	NA
Penicillin	S		31.9 \pm 0.25	29.1 \pm 0.56*	31.4 \pm 0.24	31.9 \pm 0.39	32.7 \pm 0.36	32.3 \pm 0.29
	R		13 \pm 2.51	NA	13.0 \pm 2.74	NA	NA	NA
Cephalothin	S		31.6 \pm 0.25	29.9 \pm 0.58*	31.4 \pm 0.25	30.1 \pm 0.39	32.3 \pm 0.36*	31.3 \pm 0.29
	R		0.0 \pm 2.51	0.0 \pm 2.51	0.0 \pm 1.94	NA	NA	NA
Cephadrine	S		29.5 \pm 0.25	29.6 \pm 0.58	29.5 \pm 0.25	27.7 \pm 0.39	29.1 \pm 0.36*	28.5 \pm 0.29
	R		0.0 \pm 2.51	0.0 \pm 2.51	0.0 \pm 1.94	NA	NA	NA

Table 5.7 Diameters of zones of inhibition - Continued

Macrolides /lincosamides	Erythromycin		S	25.4 ± 0.25	25.8 ± 0.58	25.4 ± 0.25	23.1 ± 0.39	23.8 ± 0.36	23.5 ± 0.29
	R	14.3 ± 1.45	12.0 ± 2.51	13.8 ± 1.37	NA	NA	NA	NA	NA
	Lincomycin		S	23.2 ± 0.39	21.5 ± 1.78	23.2 ± 0.41	NA	NA	NA
	R	18.5 ± 0.31	3.1 ± 0.59*	15.1 ± 0.30	18.1 ± 0.39	15.6 ± 0.36*	16.7 ± 0.29		
Aminoglycosides	Neomycin		S	NA	33.0 ± 2.51	33.0 ± 2.74	NA	NA	NA
	R	8.9 ± 0.24	5.8 ± 0.58*	8.5 ± 0.24	9.1 ± 0.39	11.1 ± 0.36*	10.2 ± 0.29		
	Streptomycin		S	NA	NA	NA	NA	NA	NA
	R	0.1 ± 0.24	0.5 ± 0.56	0.2 ± 0.24	9.9 ± 0.39	10.8 ± 0.36	10.3 ± 0.29		
Tetracyclines	Oxytetracycline		S	26.2 ± 0.25	27.8 ± 1.03	26.3 ± 0.26	23.4 ± 1.12	24.3 ± 1.26	23.8 ± 0.91
	R	22.0 ± 2.51	13.4 ± 0.67*	13.9 ± 0.71	21.1 ± 0.42	17.9 ± 0.38*	19.3 ± 0.31		
	Tetracycline		S	26.1 ± 0.25	26.3 ± 1.03	26.1 ± 0.26	23 ± 2.51	NA	23.0 ± 2.74
	R	21.2 ± 1.12	10.4 ± 0.67*	13.2 ± 0.63	20.5 ± 0.40	17.6 ± 0.36*	18.9 ± 0.29		
Quinolones	Enrofloxacin		S	23.9 ± 0.31	24.1 ± 0.79	24.0 ± 0.31	23.2 ± 0.70	23.5 ± 0.63	23.4 ± 0.51
	R	21.4 ± 0.40	21.6 ± 0.79	21.4 ± 0.39	21.5 ± 0.47	21.2 ± 0.44	21.3 ± 0.35		

¹Susceptible; ²Resistant; ³Not applicable; *different from mean diameter of zones of inhibition in New Zealand for the same antimicrobial/causative organism combination (P<0.05).

5.4.3 Discordant isolates

There were 173, 77, and 68 isolates of *Staph. aureus*, *Strep. uberis* and *Strep. dysgalactiae* susceptible to erythromycin, but not to lincomycin.

A small proportion of isolates of *Strep. uberis* (13/126), were resistant to penicillin and susceptible to oxacillin, being 12 from the USA and 1 from New Zealand.

5.5 Discussion

This study detected a wide variation in the susceptibility of the three common mastitis-causing organisms, *Staph. aureus*, *Strep. uberis* and *Strep. dysgalactiae* isolated from milk samples collected in New Zealand or the USA. The prevalence of resistance varied widely among the individual species. All isolates were susceptible only to amoxicillin/clavulanic acid combination. Resistance was most frequently observed to lincomycin (susceptibility of 5.9%). The susceptibility of all three tested organisms was high to most antimicrobials, except lincosamides and, in the case of streptococci, to aminoglycosides and quinolones. Additionally, a very low susceptibility to tetracyclines was found for *Strep. dysgalactiae* isolates. The susceptibility of the two streptococcal species was not identical.

Results from antimicrobial testing should be interpreted according to generally-accepted and clinically relevant guidelines after being carried out under a highly standardised protocol. There is no world-wide consensus on interpretive criteria for susceptibility testing. In the present study the SIR (susceptible, intermediate, resistant) system as recommended by CLSI was used.

The disk diffusion method is a qualitative method for testing susceptibility. In the present study the diameters of zones of inhibition were often associated with the country of origin of the isolate. The diameters of zones of inhibition can be correlated with data obtained from dilution methods for obtaining the MIC (Walker 2006), and whether the differences in the diameters of the zones of inhibition have implications in terms of clinical efficacy of the tested antimicrobials is not known. New Zealand isolates of *Staph. aureus* demonstrated smaller zones of inhibition to numerous antimicrobials. This may be evidence that corresponding MIC values should be higher. If so, it is posited that for an efficacious treatment of bovine mastitis caused by this organism in New Zealand, the dose of antimicrobial (beta-lactams, erythromycin, neomycin and tetracyclines) should be

higher than in the USA. On the other hand, for New Zealand isolates of *Strep. uberis*, the doses of antimicrobials (beta-lactams, neomycin and tetracyclines) should be lower. This area of research should be further explored to reach firm conclusions.

The organisms that were evaluated in this study are important aetiological agents in New Zealand (McDougall *et al.* 2007; Petrovski *et al.* 2009) and the USA (Erskine *et al.* 1988; Wilson *et al.* 1997). The availability of the collection of isolates in the present study allowed a comparison of the antimicrobial susceptibility of the three major mastitis-causing organisms without bias being introduced at the farm level. This is because only a single isolate per farm was tested and isolates came from various locations throughout New Zealand and the USA.

The antimicrobial compounds used in the present study were representative of different classes indicated for the treatment of mastitis, or that are of significance in human medicine. The choice of mastitis treatment products depends on availability and regulations that differ among countries. The differences in availability of products between New Zealand and the USA and, therefore, the usage of antimicrobial agents, could result in differences in the susceptibility of common mastitis-causing organisms due to different selective pressure by antimicrobials. In both countries, at the time of collection of the isolates, a limited number of antimicrobial groups was available for intramammary treatment of mastitis. In New Zealand, these included beta-lactams (amoxicillin/clavulanic acid combination, ampicillin, cloxacillin, nafcillin, penicillin, cefquinome, cefuroxime, cephalonium, cephapirin), macrolides (oleandomycin), lincosamides (lincomycin), aminoglycosides (framycetin, neomycin, streptomycin), tetracyclines (oxytetracycline) and coumarines (novobiocin). In the USA they included beta-lactams (penicillin, cephapirin, ceftiofur, amoxicillin, hetacillin and cloxacillin), macrolides (erythromycin), lincosamides (pirlimycin) and coumarines (novobiocin).

The results of the present study indicate that extrapolating the results of a particular antimicrobial to the whole class may be misleading. This may be a valid observation, or may appear to be so because of the lack of valid breakpoints for several of the key pathogen/antimicrobial combinations. The CLSI guidelines (Watts *et al.* 2008) recommend testing susceptibility to ampicillin as a class representative for an extended spectrum of penicillins, oxacillin as a similar representative for isoxazolyl penicillins and tetracycline for tetracyclines. However, the results did not support these

recommendations for the isolates of *Strep. uberis*. There were significant differences in the results between ampicillin and amoxicillin, all isoxazolyl penicillins (cloxacillin, nafcillin and oxacillin) and between tetracycline and oxytetracycline. Therefore, it is not safe to assume susceptibility unless the target organism has been tested against the specific antimicrobial compound. The guidelines for testing and interpretation of results of many mastitis-causing organisms/antimicrobial combinations are still lacking and considerable work is required before they will be available.

Discordant isolates, susceptible to erythromycin, but resistant to lincomycin, have been reported in the human literature as having decreased clinical susceptibility to both lincosamides and macrolides (Levy 2001; Levin *et al.* 2005). A lower susceptibility to lincosamides compared to macrolides has been reported previously for staphylococcal and streptococcal isolates of animal origin (Luthje and Schwarz 2007), as has a decreased susceptibility to both drug groups in isolates of environmental streptococci (Loch *et al.* 2005). Resistance to macrolides and lincosamides can result from target site modification, active efflux and enzymatic inactivation (Luthje and Schwarz 2007). All isolates of *Strep. uberis* and *Strep. dysgalactiae* resistant to lincosamides and macrolides carried an *ermB* gene (Loch *et al.* 2005; Schmitt-Van de Leemput and Zadoks 2007). Additionally, discordant isolates were carriers of the *linB* gene (Levy 2001; Schmitt-Van de Leemput and Zadoks 2007). Indiscriminate or poorly-controlled use of those antimicrobial agents in dairy cows is likely to select for streptococci carrying this gene. In support of this view, an increasing level of resistance to macrolides has recently been reported for isolates from New Zealand (Petrovski *et al.* 2011 compared to Carman and Gardner 1997). Additionally, results from the present study indicate a high level of resistance of streptococci to lincosamides. Unfortunately, the *ermB* gene from environmental streptococci is easily transmitted to other bacterial species (Roberts and Brown 1994; Hakenbeck *et al.* 1998) and to human streptococci (Martel *et al.* 2005). Therefore, the resistant isolates found in the present study may be of clinical importance, not only for treating bovine mastitis, but perhaps also for treating other pathogens such as human streptococci. Based on the findings of the present study and previous reports on the genetics of resistance to macrolides and lincosamides (Loch *et al.* 2005; Schmitt-Van de Leemput and Zadoks 2007), the use of macrolides and, particularly, lincosamides, for treatment of bovine mastitis should be discouraged and restricted.

Discordant isolates of *Strep. uberis*, that were susceptible to penicillin, but resistant to oxacillin were also found. Oxacillin resistance and penicillin susceptibility discordance has previously been reported in some isolates of *Strep. pneumoniae* (Hakenbeck *et al.* 1998). Resistance to beta-lactam isoxazolyl penicillins is due to alterations in penicillin binding proteins (PBP) (Dowson *et al.* 1994). Streptococci contain five PBPs that can be altered (Dowson *et al.* 1994; Zhao *et al.* 2000). Each particular beta-lactam antimicrobial compound has a primary target PBP (Dowson *et al.* 1994). Alterations in the particular PBP reduce the affinity for the particular antimicrobial (Dowson *et al.* 1994). In such a case, the second highest affinity PBP becomes the primary target, but the MIC is usually significantly increased (Dowson *et al.* 1994). It is possible that the second highest affinity PBP for oxacillin is actually the primary one for penicillin (Dowson *et al.* 1994). Hence, penicillin susceptibility is not necessarily decreased (Dowson *et al.* 1994). Additionally, interspecies gene transfer is possible (Liu *et al.* 1998). Fortunately, not all isolates exposed to the forced gene transfer acquired this type of resistance (Hakenbeck *et al.* 1998). In some cases of isoxazolyl penicillin resistance there is also a cross resistance to other beta-lactams, including cephalosporins (Dowson *et al.* 1994). In the present study, no *Strep. uberis* isolates resistant to oxacillin demonstrated cross-resistance to the cephalosporins tested. This may mirror the true situation or the tested cephalosporins may have different primary affinity PBPs. This type of discordance in the present study, presages possible therapeutic problems in geographic areas where the isolates came from. The most *Strep. uberis* isolates with this type of discordance in the present study originated from the USA, although a previous report from New Zealand (Petrovski *et al.* 2011) has demonstrated that such isolates also exist in New Zealand.

This type of discordance may have implications for choosing the appropriate product for treatment. Currently, mastitis treatment is initiated before the identity and susceptibility of mastitis-causing organisms are known. In the near future however, the identity of the organism is likely to be known immediately as a cow-side pathogen identification tests are developed. The treatment of bovine mastitis caused by streptococci, particularly *Strep. uberis*, with isoxazolyl penicillins should be discouraged nationally and internationally. They should be reserved for treatment of beta-lactamase producing staphylococci. This procedure is likely to extend the clinical life of these antimicrobials for effective treatment of bovine mastitis.

Likewise, the intrinsic resistance of streptococci to aminoglycosides does not support their use for the treatment of bovine mastitis. Their use, combined with beta-lactams (*i.e.* penicillin/streptomycin or penicillin/neomycin) may be only justified in treatment of *Staph. aureus*, in which approximately 18% of isolates resistant to penicillin may be susceptible to streptomycin or neomycin. Whether this is of clinical significance is debatable. Combinations of isoxazolyl penicillins with aminoglycosides will provide no improvement in treating streptococci. The findings of this study do not support the use of aminoglycosides in the treatment of bovine mastitis.

The susceptibility of *Streptococcus* spp. isolates, with the exception of isolates of *Strep. uberis* from New Zealand, to tetracyclines was less than for other antimicrobials. This, coupled with unfavourable pharmacokinetics (Barza *et al.* 1975; Fang and Pyorala 1996; Jung *et al.* 1997) and decreased activity in milk (Owens and Watts 1987; Fang and Pyorala 1996; Jung *et al.* 1997) renders them ineffective for treating bovine mastitis, particularly when caused by streptococci.

The commercial availability of antibiotics in intramammary products appeared to be unrelated to the susceptibility of tested organisms. On the other hand, there is very little information available about the volumes of each of these antibiotics/intramammary formulations that are actually used in treating animals with mastitis. This would require knowledge of the use of a specific antimicrobial at the farm level; data which were unavailable in the present collection of isolates. Previous reports have indicated that the volume of use of antimicrobials is not closely related to development of resistance (Berghash *et al.* 1983; Tikofsky *et al.* 2003; Pol and Ruegg 2007). Therefore it seems that the use of intramammary antimicrobials is only one, and not necessarily the most important, factor causing changes in the susceptibility pattern of a particular organism.

5.6 Conclusion

The susceptibility of the tested mastitis-causing organisms to the most antimicrobials in common use for treatment of bovine mastitis in both countries was high, except to lincosamides. Beta-lactam antimicrobials are still the preferred treatments for clinical mastitis caused by the tested organisms. The use of isoxazolyl penicillins should be avoided when *Strep. uberis* is the causative agent. Tetracyclines, quinolones and aminoglycosides should be avoided for treatment of streptococcal mastitis. Differences in

the susceptibility and in the diameters of zones of inhibition between the two countries support the value of national surveys to test the susceptibility of mastitis causing organisms, possibly using more definitive techniques. Testing class representatives or susceptibility at a genus level may not be appropriate for mastitis-causing organisms, particularly for streptococci.

5.7 Acknowledgments

The study was financially supported by *Bomac Laboratories Ltd.* The staff at *Gribbles Veterinary Laboratories* Auckland, Christchurch, Dunedin, Hamilton, and Palmerston North, New Zealand and the *Veterinary Diagnostic Laboratory*, Saint Paul, University of Minnesota, Minnesota, USA are thanked for their contribution in the study. KR Petrovski's position at Massey University is supported by *Bomac a company of Bayer Ltd.*

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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Kiro R Petrovski

Name/Title of Principal Supervisor: Prof Norman B Williamson

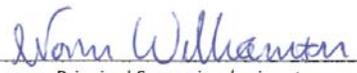
Name of Published Paper: Susceptibility to antimicrobials of mastitis-causing *Staphylococcus aureus*, *Streptococcus uberis* and *Strep. dysgalactiae* from New Zealand and the USA as assessed by the disk diffusion test

In which Chapter is the Published Work: Chapter 5

What percentage of the Published Work was contributed by the candidate: 70%


Candidate's Signature

11 July 2011
Date


Principal Supervisor's signature

14/7/2011
Date

Chapter 6

Correlation of agar disk diffusion and broth microdilution methods when testing the antimicrobial susceptibility of *Staphylococcus aureus* and streptococci isolated from bovine milk samples collected in New Zealand

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6. Correlation of the antimicrobial susceptibility of *Staphylococcus aureus* and streptococci isolated from bovine milk samples collected in New Zealand when tested by the agar disk diffusion and broth microdilution methods

6.1 Abstract

AIMS: This study assessed the inter-test diagnostic agreement and correlation of the results of the agar disk diffusion and broth microdilution methods for testing the susceptibility to antimicrobial drugs of isolates of *Staphylococcus aureus* and streptococci from bovine milk samples collected in New Zealand.

METHODS: Isolates were tested against the following antimicrobials: ampicillin, cloxacillin, enrofloxacin, neomycin, oxytetracycline and penicillin, following the guidelines of the Clinical and Laboratory Standards Institute. The inter-test categorical agreement and the correlation between the results obtained by the two tests were estimated.

RESULTS: The inter-test categorical agreement was high (92.6%), varying from 97.2% for neomycin, cloxacillin (96.3%), oxytetracycline (94.1%), ampicillin (93.7%), penicillin (90.7%) and enrofloxacin (83.6%). The inter-test categorical agreement was 98.5%, 78.0% and 77.5% for *Staph. aureus*, *Streptococcus dysgalactiae* and *Strep. uberis*.

The correlation between the zones of inhibition and the minimal inhibitory concentrations was $R^2=0.23$ (slope -0.33 ; $P<0.001$) when all isolates were included and varied among antimicrobial/organism combination, being higher for *Staph. aureus* than for either of the streptococci.

CONCLUSION: Variation in the inter-test categorical agreement and inter-test correlation depended on the combination of antimicrobial tested and genus of the isolated organism. New breakpoints for the antimicrobials used for mastitis treatment for common mastitis-causing organisms at a species level are required. When reporting the results of the agar disk diffusion test, laboratories and authors should include the size of zones of inhibition observed.

CLINICAL RELEVANCE: Antimicrobial susceptibility testing carried out by commercial veterinary laboratories is commonly undertaken using agar disk diffusion. While there is a high correlation between the inferences drawn from disk diffusion and microdilution techniques, for some pathogen/antimicrobial combinations significant misclassifications appears to be occurring. Hence results based on disc diffusion should be interpreted with care, particularly in the face of unexpected clinical outcomes.

KEY WORDS: Mastitis, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, susceptibility, antimicrobial.

CLSI – Clinical and Laboratory Standards Institute; EUCAST – European Committee on Antimicrobial Susceptibility Testing; I – Intermediate Susceptibility; MIC – Minimal Inhibitory Concentrations; S – Susceptible; R – Resistant.

6.2 Introduction

Routine susceptibility testing in commercial veterinary laboratories generally uses the agar disk diffusion method, which is simple, repeatable between laboratories, flexible in the type and number of antimicrobials that can be tested and has low cost. The zones of inhibition obtained as the outcome of the test are interpreted using breakpoints established by the Clinical and Laboratory Standards Institute (CLSI), and/or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), or by using breakpoints extrapolated from similar antimicrobial/causative organism combinations. Results reported to a submitting veterinarian are that organisms are susceptible, intermediate or resistant (S, I, R) and are often used as a guideline for selection of treatment. Species-related breakpoints for zones of inhibition are used for categorising the susceptibility of causative organisms. These are derived from the correlation of minimal inhibitory concentrations (MICs) and the zones of inhibition. Susceptibility categories divide each species that does not possess intrinsic resistance, into a fully susceptible population and isolates that have some acquired low- or high-grade resistance (Ringertz *et al.* 1997; Turnidge and Paterson 2007).

Clinical breakpoints are used in the interpretation of the susceptibility of a particular organism to individual antimicrobial compounds (Jorgensen 2004; Turnidge and Paterson 2007). Strains that have developed resistance to an antimicrobial usually have a 2-log-normal distribution of MICs (Turnidge and Paterson 2007). This finding is

common in causative organisms with a single resistance mechanism and which has a bimodal distribution of MICs (Turnidge and Paterson 2007). Increased accuracy in clinical categorisation has been reported in the literature on bacterial isolates from humans recently due to the availability of appropriate breakpoints for various clinical conditions. The improved accuracy is based on research in susceptibility, pharmacokinetics and clinical efficacy for various antimicrobial/causative organism combinations.

It has been postulated that there is an inverse relationship between the results of agar disk diffusion and broth microdilution methods (Watson *et al.* 1991). Therefore, routine laboratory susceptibility testing method can be translated into expected mastitis treatment outcomes using the pharmacokinetic/pharmacodynamic approach. Some studies have been undertaken to examine the agreement in the categorical interpretation of various susceptibility testing methods for mastitis-causing organisms (Ferreiro and Biberstein 1978; Myllys *et al.* 1992; Schlegelova *et al.* 2001; Giannechini *et al.* 2002; Klement *et al.* 2005), but despite these studies, relatively little is known about the prevalence of resistance in mastitis-causing organisms to various antimicrobial compounds, nor about the cross-validation of the different methods of evaluating susceptibility. Hence, there appears to be a need to develop feasible and reliable methods for screening the susceptibility of mastitis-causing organisms, particularly in the context of validating appropriate breakpoints between categories of antimicrobial susceptibility.

This study aimed to assess the inter-test diagnostic agreement of the results of categorical interpretation criteria between agar disk diffusion and broth microdilution antimicrobial susceptibility testing methods, based on pre-set breakpoints. It further evaluated the correlation between the size of zones of inhibition and MIC levels for isolates of *Staphylococcus aureus* and streptococci from bovine milk samples collected in New Zealand.

6.3 Materials and methods

Bacterial isolates from milk samples that had been submitted to commercial diagnostic laboratories for routine testing of cows with suspected clinical and subclinical mastitis throughout New Zealand and from samples collected during clinical studies (*Cognosco*, Morrinsville, New Zealand) were received. For the isolates from commercial laboratories, single isolates of a particular species of bacteria per farm were used to test their

susceptibility to various antimicrobials. For the isolates from clinical studies, multiple isolates per farm were used to test their susceptibility to the same antimicrobials.

The disk-diffusion test was carried out using commercially available test disks (*Oxoid*, Auckland, New Zealand). Customised commercial microtitre plates (Sensititre, *Trek Diagnostics*, UK) were used for broth microdilution testing of the MIC. Only isolates for which there were data regarding zones of inhibition and where MICs were available were included. Categorical interpretation followed the guidelines for bacteria isolated from animals of the CLSI (Watts *et al.* 2008). There were 364, 41, and 102 isolates of *Staph. aureus*, *Streptococcus dysgalactiae* and *Strep. uberis* tested for susceptibility to ampicillin, cloxacillin, enrofloxacin, oxytetracycline and penicillin.

The inter-test categorical agreement in the interpretation criteria of susceptibility (S, I and R) for the zones of inhibition (mm) and minimal inhibitory concentrations ($\mu\text{g}/\text{mL}$) of each antimicrobial/causative organism combination was assessed following rules established for the error-bounded method (Metzler and DeHaan 1974; Brunden *et al.* 1992):

1. Agreement (coded as 1) was when the categorisations were the same in both tests.
2. Disagreement (coded as 0) was when the categorisation conflicted between tests (*i.e.* Test 1: susceptible, Test 2: resistant (major error); Test 1: susceptible or resistant, Test 2: intermediate (minor error)).

The correlation coefficient (R^2) and the slope of the linear regression line for the sizes of zones of inhibition *vs.* the MIC_{90} values for a particular antimicrobial/causative organism combination were calculated. Linear regression was executed using the General Linear Model (GLM) procedure of SAS (Statistical Analysis System, *SAS Institute Inc.*, Cary, NC, USA 2003), version 9.2.

Current interpretive criteria for bacteria isolated from animals based on the CLSI breakpoints (Watts *et al.* 2008) are presented in Table 6.1. None of the antimicrobial/causative organism combinations had been specifically validated previously for mastitis-causing organisms.

Table 6.1. Interpretive criteria for bacteria isolated from animals (if not stated otherwise based on the Clinical and Laboratory Standards Institute, 2008)

Antimicrobial	Interpretive criteria	<i>Staphylococcus aureus</i>		Streptococci	
		DDT (mm)	MIC ₉₀ (µg/mL)	DDT (mm)	MIC ₉₀ (µg/mL)
Ampicillin	S	≥20	≤0.25	≥26	≤0.25
	I	-	-	19-25	0.5-4
	R	≤19	≥0.5	≤18	≥8
Cloxacillin ¹	S	≥13	≤2	≥13	≤2
	I	11-12	-	11-12	-
	R	≤10	≥4	≤10	≥4
Enrofloxacin	S	≥23	≤0.5	≥23	≤0.25
	I	17-22	1-2	17-22	0.5-1
	R	≤16	≥4	≤16	≥2
Neomycin ²	S	≥15	≥2	≥15	≥2
	I	13-14	4	13-14	4
	R	≤12	≤8	≤12	≤8
Oxytetracycline ³	S	≥19	≤4	≥23	≤2
	I	15-18	8	19-22	4
	R	≤14	≥16	≤18	≥8
Penicillin	S	≥29	≤0.12	≥24	≤0.12
	I	-	-	-	0.25-2
	R	≤28	≥0.25	≤23	≥4

¹interpretation criteria for oxacillin; ²Disk diffusion Test (DDT) breakpoint from Bauer *et al.* 1966, Minimal Inhibitory concentrations (MIC) breakpoint from Klement *et al.* 2005; ³interpretation criteria for tetracycline

The graphic presentation of the distribution of the susceptibility results used the scheme given in Figure 6.1.

When all isolates were susceptible or there was no intermediate range in the interpretive criteria, the minor errors were not considered (Pengov and Ceru 2003) and the graphic presentation omits the intermediate range.

The acceptable rate of errors (minor and major) in the inter-test categorical agreement was $\leq 5\%$ (Brunden *et al.* 1992; Turnidge and Paterson 2007). The level of significance of the correlation slope was set at $P < 0.05$.

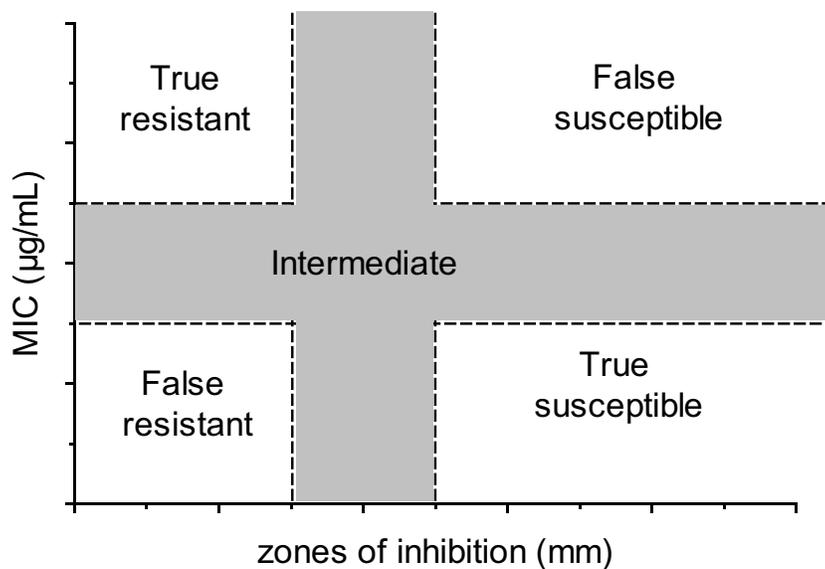


Figure 6.1. Schema of the graphic presentation of each antimicrobial/isolates of causative organism susceptibility testing outcome

6.4 Results

6.4.1 Overall

There were 6,084 antimicrobial/causative organism tests. The susceptibility was 2509/3042 (82.5%) and 2451/3042 (80.6%) based on agar disk diffusion and broth microdilution method respectively. Inter-test categorical agreement occurred in 2817/3042 (92.6%) results. Major and minor classification errors occurred for 48 (1.6%) and 177 (5.8%) of isolates, respectively.

The inter-test categorical agreement for neomycin was 97.2% (493/507), cloxacillin 96.3% (488/507), oxytetracycline 94.1% (477/507), ampicillin 93.7% (475/507), penicillin 90.7% (460/507) and enrofloxacin 83.6% (424/507). The inter-test categorical agreement for *Staph. aureus* was 98.5% (2151/2184) and for streptococci 77.6% (666/858), of which for *Strep. dysgalactiae* it was 78.0% (192/246) and for *Strep. uberis* 77.5% (474/612).

An inverse correlation existed between the two tests for all isolates ($R^2=0.23$; slope -0.33 ; $P<0.001$). The level of this correlation differed depending on the antimicrobial/causative organism combination (Table 6.2), being higher for *Staph. aureus* than for streptococci.

Table 6.2. Average sizes of inhibition at agar disk diffusion (mm ± SE) compared to microdilution test results (µg/mL) for various antimicrobial and causative organism combinations

Antimicrobial	MIC ₉₀	Zone of inhibition		
		<i>Staphylococcus aureus</i>	Streptococci	All
Ampicillin	0.12	34.7 ± 0.22	29.9 ± 0.28	33.2 ± 0.21
	0.25	32.5 ± 0.68	29.1 ± 0.63	31.0 ± 0.59
	0.5	21.8 ± 1.69	29.5 ± 0.72	27.9 ± 0.90
	1	16.0 ± 0.41	30.3 ± 1.62	16.6 ± 0.47
	2	15.8 ± 0.82	19.0 ± 2.81	16.0 ± 0.93
	4	13.5 ± 1.69	-	13.5 ± 1.97
	8	11.5 ± 2.39	-	11.5 ± 2.79
	16	14.0 ± 2.39	-	14.0 ± 2.79
Cloxacillin	0.06	-	25.1 ± 0.88	25.1 ± 1.04
	0.12	29.7 ± 0.52	25.1 ± 0.45	26.9 ± 0.21
	0.25	28.2 ± 0.15	23.8 ± 0.37	27.4 ± 0.18
	0.5	27.9 ± 0.18	23.8 ± 0.37	27.0 ± 0.20
	1	27.5 ± 1.07	25.5 ± 1.65	26.8 ± 1.12
	2	-	21.6 ± 0.83	21.6 ± 0.97
	4	20.0 ± 1.51	20.8 ± 0.58	20.7 ± 0.65
	8	-	8.5 ± 1.66	8.5 ± 1.94
Enrofloxacin	0.12	27.0 ± 0.08	-	27.0 ± 0.08
	0.25	26.4 ± 0.19	24.0 ± 1.38	26.4 ± 0.20
	0.5	26.0 ± 1.02	22.3 ± 0.21	22.5 ± 0.22
	1	25.0 ± 0.83	23.0 ± 0.14	23.0 ± 0.15
	2	26.0 ± 1.44	21.8 ± 0.56	22.4 ± 0.55
Neomycin	0.12	21.0 ± 0.90	9.0 ± 0.92	17.0 ± 1.14
	0.25	19.7 ± 0.22	-	19.7 ± 0.36
	0.5	19.7 ± 0.09	-	19.7 ± 0.14
	1	19.6 ± 0.14	-	19.7 ± 0.22
	2	20.0 ± 0.29	-	20.0 ± 0.46
	4	20.7 ± 0.74	11.5 ± 0.65	17.0 ± 0.89
	8	-	9.5 ± 0.65	9.5 ± 1.40
	16	21.0 ± 0.64	9.3 ± 0.21	11.3 ± 0.41
	32	20.8 ± 0.64	9.0 ± 0.17	10.4 ± 0.35
	64	20.0 ± 1.28	8.9 ± 0.10	9.0 ± 0.21
Oxytetracycline	0.5	23.2 ± 0.19	26.3 ± 0.23	24.9 ± 0.16
	1	22.9 ± 0.09	24.5 ± 0.42	23.0 ± 0.11
	2	23.0 ± 0.63	21.8 ± 0.44	22.1 ± 0.36
	4	-	22.2 ± 0.57	22.2 ± 0.53
	8	-	22.0 ± 2.07	22.0 ± 1.92
	64	-	20.0 ± 2.07	20.0 ± 1.92
Penicillin	0.03	36.8 ± 0.25	29.9 ± 0.28	35.5 ± 0.24
	0.06	35.6 ± 0.72	29.1 ± 0.63	34.7 ± 0.71
	0.12	29.2 ± 1.67	29.5 ± 0.72	31.6 ± 0.79
	0.25	24.9 ± 1.42	30.3 ± 1.62	29.6 ± 0.79
	0.5	14.9 ± 1.18	19.0 ± 2.81	24.2 ± 0.90
	1	15.8 ± 0.54	-	16.5 ± 0.61
	2	14.9 ± 0.71	-	14.9 ± 0.82
	4	20.7 ± 2.39	-	20.7 ± 2.51
	8	-	-	12.5 ± 1.77

6.4.2 Ampicillin

Isolates of *Staph. aureus* demonstrated a bimodal distribution on disk-diffusion testing for susceptibility to ampicillin. Ninety-seven (26.7%) isolates of *Staph. aureus* were ampicillin-resistant based on the MIC results. The inter-test categorical agreement was high (98.1%, 357/364), but all isolates with disagreement (n=7, 1.9%) were classified as major errors (Figure 6.2A).

Most isolates of streptococci were susceptible to ampicillin. Based on the MIC results, 3/41 (7.3%) and 16/102 (15.7%) isolates of *Strep. dysgalactiae* and *Strep. uberis*, respectively were of intermediate susceptibility to ampicillin. The inter-test categorical agreement for both streptococci was 82.5% (118/143). All 25 isolates with disagreement (n=25, 17.5%) were classified as minor errors (Figure 6.2B). Inter-test categorical agreements for *Strep. dysgalactiae* and *Strep. uberis* were 87.8% (36/41) and 80.4% (82/102), respectively.

An inverse correlation existed between the results of the two tests (Table 6.3) for all isolates to ampicillin ($R^2=0.24$ slope -3.06 ; $P<0.001$; Table 6.2). However, the slope was not significant for isolates of *Strep. dysgalactiae* (Table 6.2).

Table 6.3. Correlation parameters for the zones of inhibition measured using the agar disk diffusion method with the MIC obtained by the broth microdilution method for various antimicrobial/causative organism combinations

Antimicrobial	Organism	R ²	Intercept	Slope
All	All	0.23	27.20 ± 0.13*	-0.33 ± 0.01*
	<i>Staphylococcus aureus</i>	0.28	26.75 ± 0.15*	-0.32 ± 0.01*
	Streptococci	0.06	28.42 ± 0.26*	-0.56 ± 0.08*
	<i>Streptococcus dysgalactiae</i>	0.05	29.14 ± 0.48*	-0.85 ± 0.23*
	<i>Strep. uberis</i>	0.06	28.15 ± 0.31*	-0.52 ± 0.08*
Ampicillin	All	0.24	30.98 ± 0.32*	-3.06 ± 0.25*
	<i>Staph. aureus</i>	0.25	31.28 ± 0.44*	-3.11 ± 0.29*
	Streptococci	0.05	30.24 ± 0.33*	-2.82 ± 1.07*
	<i>Strep. dysgalactiae</i>	0.00	30.23 ± 0.53*	-0.69 ± 2.84
	<i>Strep. uberis</i>	0.05	30.10 ± 0.43*	-2.81 ± 1.24#
Cloxacillin	All	0.28	27.83 ± 0.15*	-2.02 ± 0.15*
	<i>Staph. aureus</i>	0.08	28.87 ± 0.18*	-2.14 ± 0.37*
	Streptococci	0.40	24.71 ± 0.24*	-1.36 ± 0.14*
	<i>Strep. dysgalactiae</i>	0.00	25.12 ± 0.53*	-1.29 ± 3.03
	<i>Strep. uberis</i>	0.39	24.46 ± 0.33*	-1.30 ± 0.16*
Enrofloxacin	All	0.47	27.20 ± 0.10*	-4.14 ± 0.20*
	<i>Staph. aureus</i>	0.02	27.12 ± 0.12*	-1.61 ± 0.56#
	Streptococci	0.00	22.60 ± 0.34*	0.15 ± 0.36
	<i>Strep. dysgalactiae</i>	0.06	22.58 ± 0.40*	-0.82 ± 0.53
	<i>Strep. uberis</i>	0.00	23.31 ± 0.49*	-0.31 ± 0.48
Neomycin	All	0.74	19.34 ± 0.13*	-0.18 ± 0.00*
	<i>Staph. aureus</i>	0.01	19.72 ± 0.07*	0.03 ± 0.02
	Streptococci	0.04	9.49 ± 0.21*	-0.01 ± 0.00#
	<i>Strep. dysgalactiae</i>	0.05	9.49 ± 0.29*	-0.01 ± 0.01
	<i>Strep. uberis</i>	0.04	9.70 ± 0.36*	-0.01 ± 0.01#
Oxytetracycline	All	0.02	23.59 ± 0.10*	-0.10 ± 0.03#
	<i>Staph. aureus</i>	0.00	23.35 ± 0.32*	-0.45 ± 0.34
	Streptococci	0.07	25.06 ± 0.23*	-0.13 ± 0.04#
	<i>Strep. dysgalactiae</i>	0.03	21.49 ± 0.18*	-0.02 ± 0.02
	<i>Strep. uberis</i>	0.04	26.70 ± 0.30*	-0.70 ± 0.33#
Penicillin	All	0.37	33.13 ± 0.33*	-5.15 ± 0.30*
	<i>Staph. aureus</i>	0.38	33.50 ± 0.45*	-5.60 ± 0.37*
	Streptococci	0.38	32.21 ± 0.18*	-2.30 ± 0.25*
	<i>Strep. dysgalactiae</i>	0.05	31.62 ± 0.31*	-1.95 ± 1.34
	<i>Strep. uberis</i>	0.46	32.26 ± 0.21*	-2.41 ± 0.26*

*P≤0.001; #P between <0.05 and P=0.002

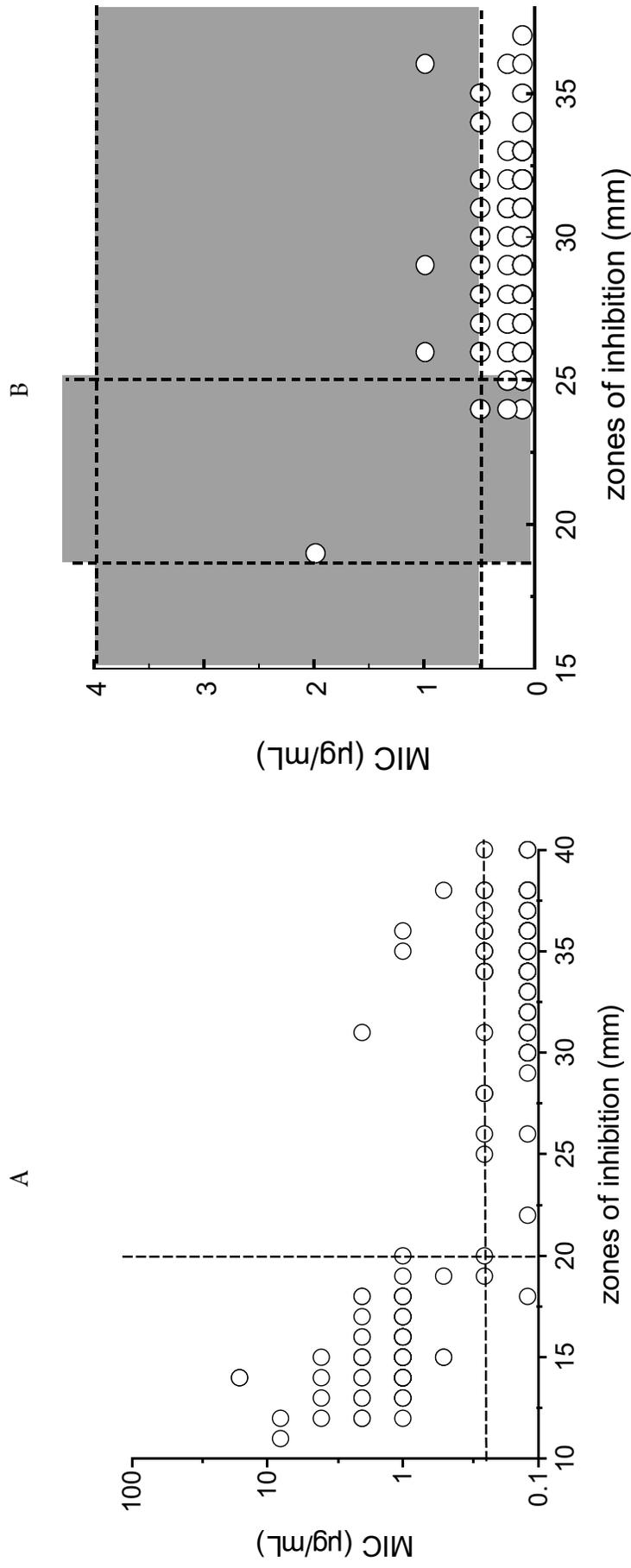


Figure 6.2. Distribution of susceptibility results of isolates of *Staphylococcus aureus* (A) or streptococci (B) to ampicillin. The Y-axis on Figure 2A is on a log-scale

6.4.3 Cloxacillin

All isolates of *Staph. aureus* were classified as susceptible to cloxacillin on the basis of disk-diffusion results and 99.5% (362/364) were also classified as susceptible on the basis of MIC results. The two isolates (0.5%), for which there was no agreement between the two methods, were classified as major errors (Figure 6.3A).

Most of the isolates of streptococci were susceptible to cloxacillin (126/143, 88.1%) based on MIC results. The balance of streptococcal isolates (17/173, 11.9%), which were resistant, were all *Strep. uberis* (83.3%; 17/102). The resistant isolates were all in disagreement with the disk-diffusion results and were categorised as major errors (Figure 6.3B).

An inverse correlation existed between the susceptibility results to cloxacillin of the two tests (Table 6.2) for all isolates ($R^2=0.28$ slope -2.02 ; $P<0.001$; Table 6.3). However, the slope was not significant for isolates of *Strep. dysgalactiae* (Table 6.3).

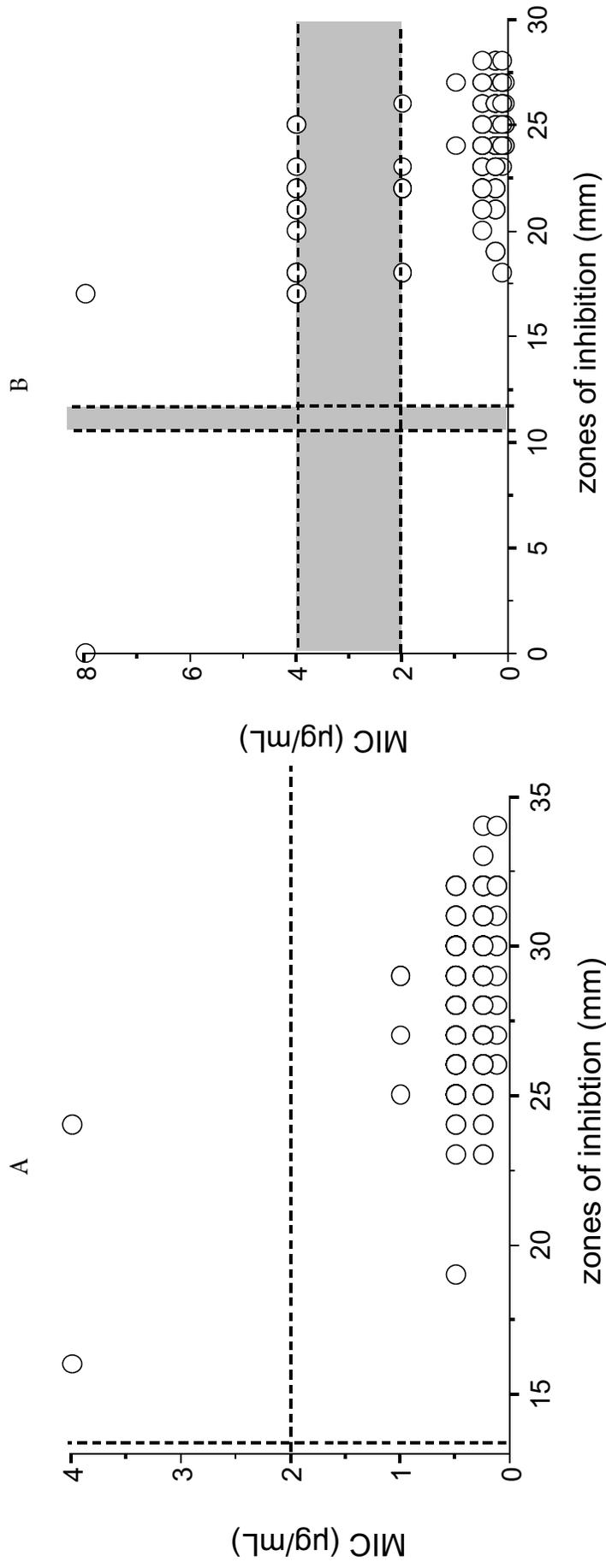


Figure 6.3. Distribution of susceptibility results of isolates of *Staphylococcus aureus* (A) or streptococci (B) to cloxacillin

6.4.4 Enrofloxacin

Most of isolates of *Staph. aureus* (360/364, 98.9%) were susceptible to enrofloxacin based on the MIC results and 4 (1.1%) were of intermediate susceptibility. Disagreement between the two tests was present in 3/364 (0.8%) isolates; all of these were categorised as minor errors (Figure 6.4A).

Most the isolates of streptococci (136/142, 95.1%; *Strep. dysgalactiae* (n=40, 97.6%); *Strep. uberis* (n=96, 94.1%)), were classed as having intermediate susceptibility to enrofloxacin on the MIC results. However, only 63/143 (44.1%) isolates showed agreement between the two tests. Of the isolates of streptococci for which the tests disagreed, 77/143 (53.8%) were minor errors and 3/143 (3.1%) were major errors (Figure 6.4B). The inter-test categorical agreement for *Strep. dysgalactiae* was 65.9% (27/41) and for *Strep. uberis* was 35.3% (36/102). The proportion of minor errors for isolates of *Strep. dysgalactiae* was 34.1% (14/41), while for *Strep. uberis* it was 61.8% (63/102). There were also 3 *Strep. uberis* isolates (2.9%) classified as major errors.

An inverse relationship existed between the results of the two tests (Table 6.4) for all isolates to enrofloxacin ($R^2=0.47$ slope -4.14 ; $P<0.001$; Table 6.2). However, the slope was not significant for isolates of streptococci (Table 6.3).

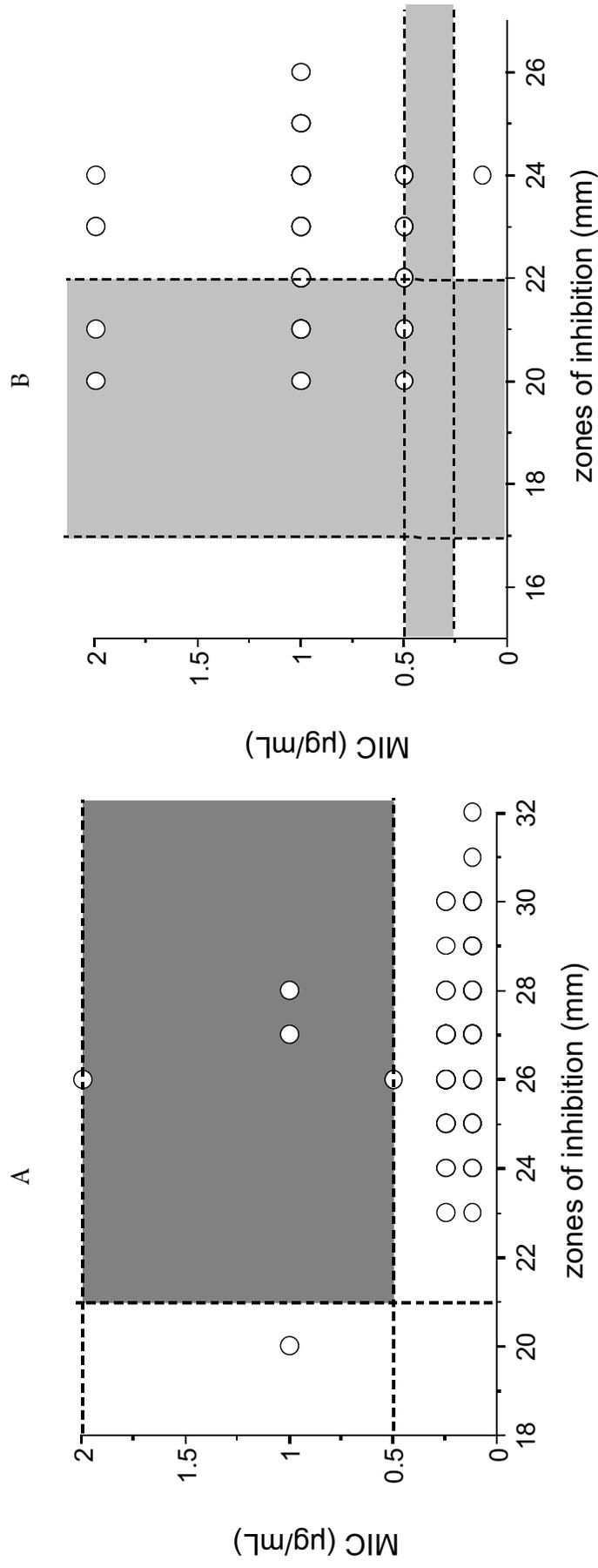


Figure 6.4. Distribution of susceptibility results of isolates of *Staphylococcus aureus* (A) or streptococci (B) to enrofloxacin

6.4.5 Neomycin

Most of the isolates of *Staph. aureus* (352/364, 96.7%) were susceptible, 3 (0.8%) were of intermediate susceptibility and 9 (2.5%) were resistant to neomycin based on the MIC results. There were 12/364 (3.3%) isolates in which the two tests were in disagreement, of which 9 (2.5%) and 3 (0.8%) were categorised as major and minor errors, respectively (Figure 6.5A).

Almost all the isolates of streptococci (142/143, 99.3%; *Strep. dysgalactiae* (n=41, 100.0%), *Strep. uberis* (n=101, 99.0%)) were resistant to neomycin based on the MIC results. In 2/143 (1.4%) isolates of streptococci the two tests were in disagreement (Figure 6.5B). The inter-test categorical agreement for *Strep. dysgalactiae* was 2.4% (1/41; classified as a minor error) and for *Strep. uberis* was 2.0% (2/102). One isolate each of the *Strep. uberis* (1.0%) were classified as minor and major errors.

An inverse correlation existed between the results of the two tests (Table 6.3) for all isolates to neomycin ($R^2=0.74$ slope -0.18 ; $P<0.001$; Table 6.2). However, the slope was not significant for isolates of *Staph. aureus* and *Strep. dysgalactiae* (Table 6.2).

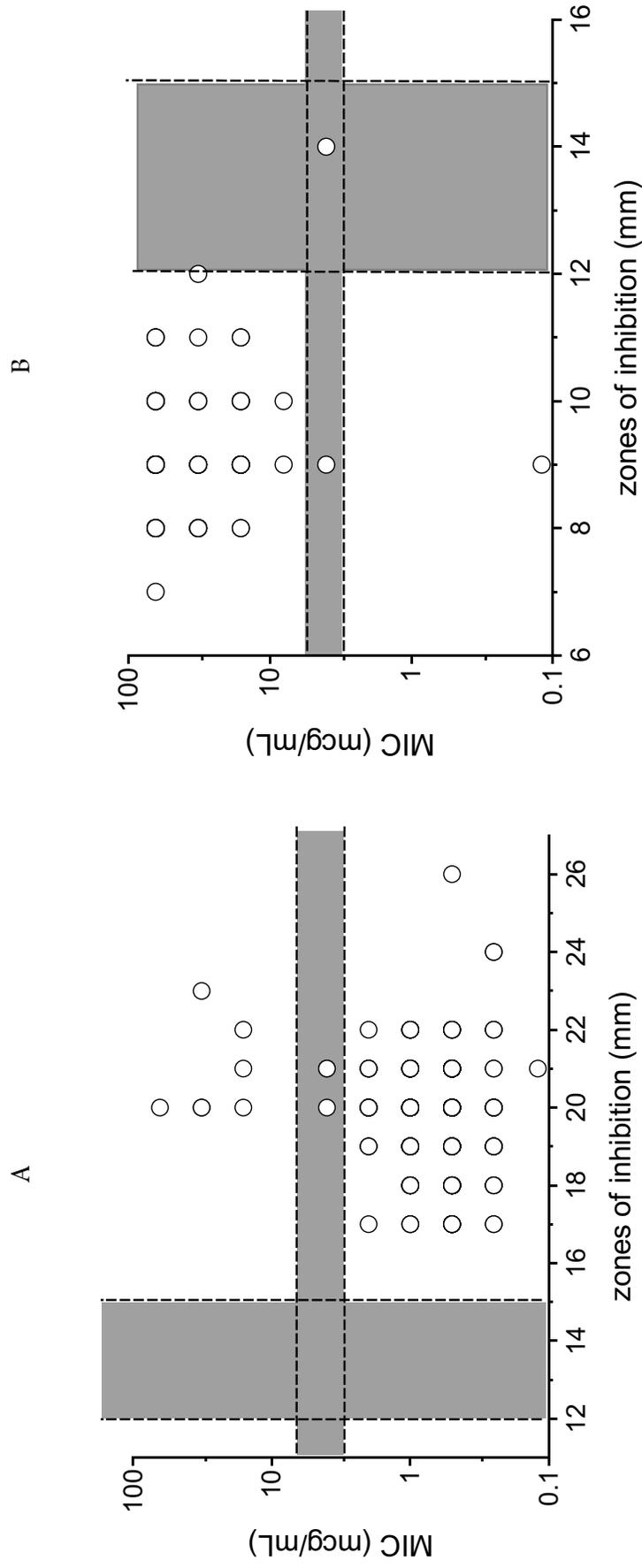


Figure 6.5. Distribution of susceptibility results of isolates of *Staphylococcus aureus* (A) or streptococci (B) to neomycin. The Y-axis is on a log-scale

6.4.6 Oxytetracycline

All isolates of *Staph. aureus* (n=364, 100%) were classified as susceptible to oxytetracycline in both tests and no errors in the inter-test categorical agreement were observed (Figure 6.6A).

Most isolates of streptococci (128/143, 85.1%; *Strep. dysgalactiae* (n=28, 68.3%); *Strep. uberis* (n=100, 98.0%)) were susceptible to oxytetracycline based on the MIC results. In 30/143 (21.0%) isolates of streptococci the two tests were in disagreement classed as minor errors (Figure 6.6B). The inter-test categorical agreement for *Strep. dysgalactiae* and *Strep. uberis* were 34.1% (14/41) and 97.1% (99/102), respectively. The proportion of minor errors for isolates of *Strep. dysgalactiae* was 65.9% (27/41), while for *Strep. uberis* it was 2.9% (3/102).

A small inverse correlation existed between the results of the two tests (Table 6.3) for all isolates to oxytetracycline ($R^2=0.02$ slope -0.10 ; $P=0.002$; Table 6.2). However, the slope was not significant for isolates of *Strep. dysgalactiae* (Table 6.2).

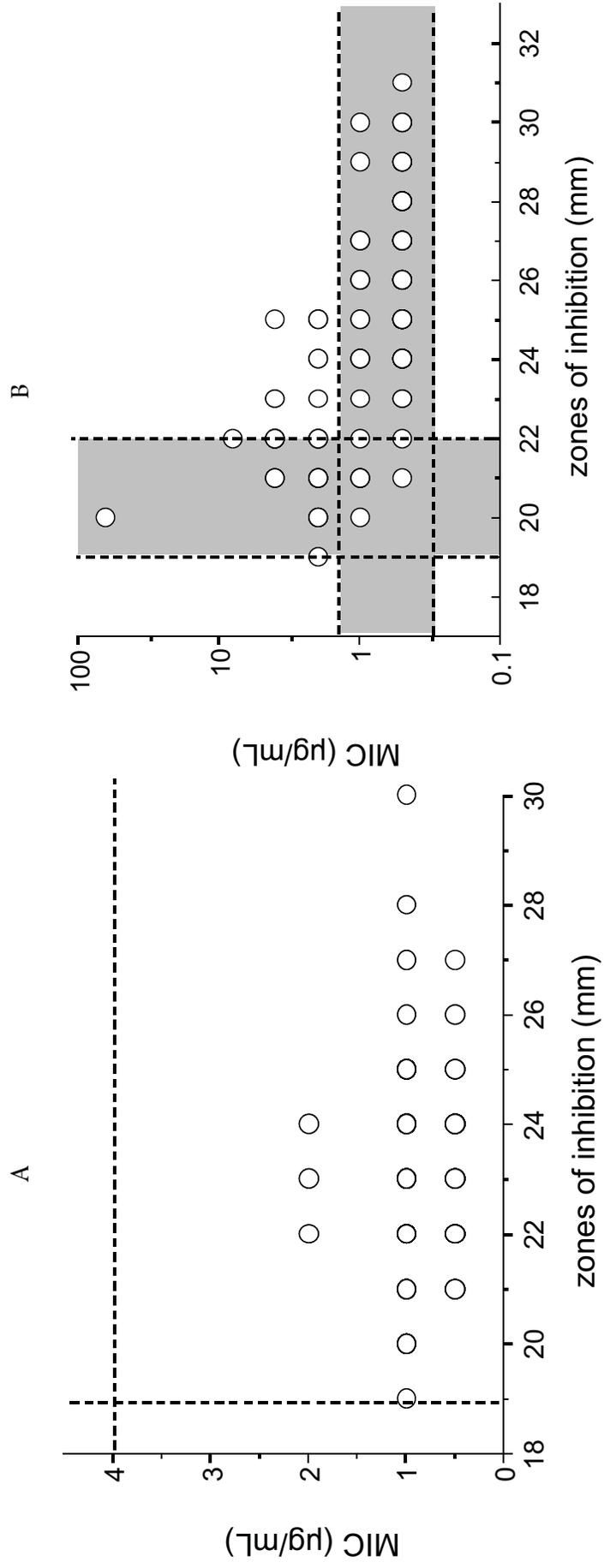


Figure 6.6. Distribution of susceptibility results of isolates of *Staphylococcus aureus* (A) or streptococci (B) to oxytetracycline. The Y-axis of 6B is on a log-scale

6.4.7 Penicillin

The isolates of *Staph. aureus* tested for susceptibility to penicillin demonstrated a bimodal distribution in the size of inhibition zones on disk-diffusion testing (Figure 6.7A). There were 263/364 (72.3%) isolates of *Staph. aureus* that were susceptible to penicillin based on the MIC results. The inter-test categorical agreement was high (97.5%, 355/364), but all isolates with disagreement (n=9; 2.5%) were classified as major errors.

Streptococci were moderately susceptible to penicillin: based on MIC results, only 104/143 (72.7%) streptococci, of which 34/41 (82.3%) *Strep. dysgalactiae* and 70/102 (68.6%) *Strep. uberis*, were susceptible to penicillin. In 105/143 (73.4%) isolates of streptococci the two tests were in agreement. All isolates of streptococci with disagreement (n=38; 26.6%) were classed as minor errors (Figure 6.7B). The inter-test categorical agreement for *Strep. dysgalactiae* was 82.9% (34/41) and for *Strep. uberis* was 69.6% (71/102). The proportion of minor errors for isolates of *Strep. dysgalactiae* was 17.1% (7/41), while for *Strep. uberis* it was 30.4% (31/102).

An inverse relationship existed between the results of the two tests (Table 6.3) for all isolates to ampicillin ($R^2=0.37$ slope -5.15 ; $P<0.001$; Table 6.2). However, the slope was not significant for isolates of *Strep. dysgalactiae* (Table 6.2).

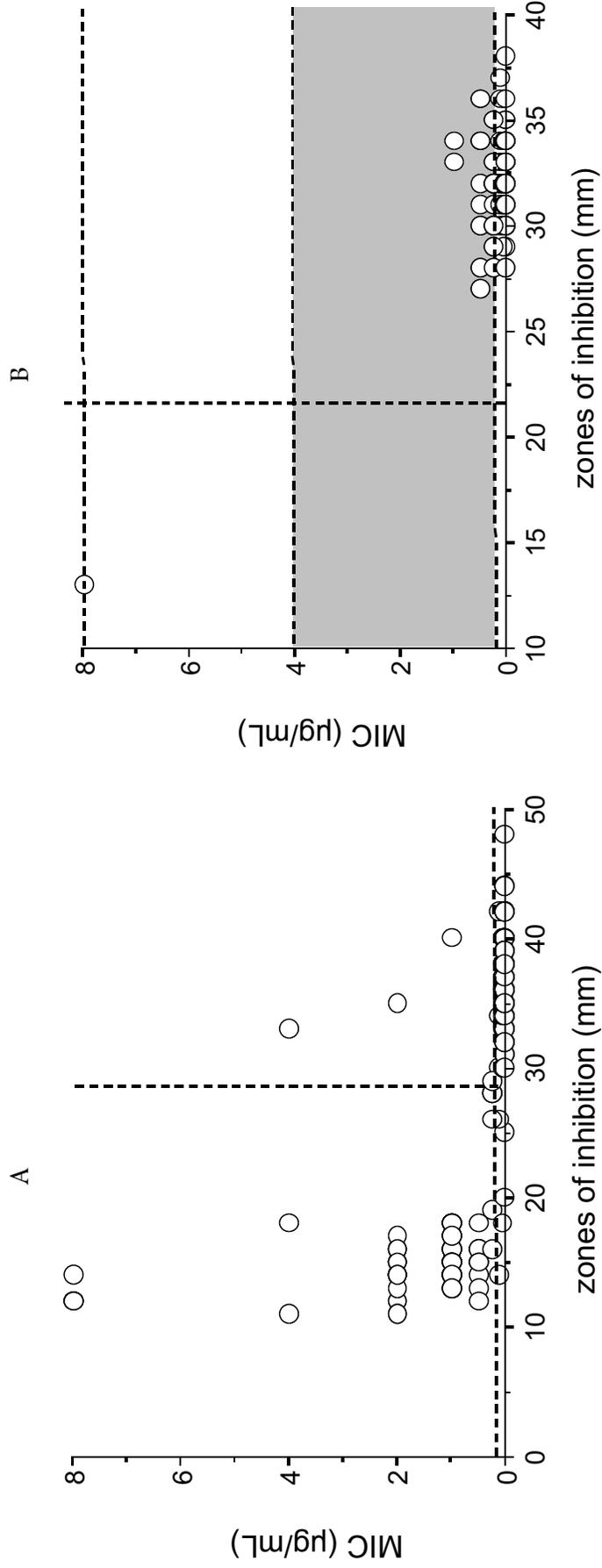


Figure 6.7. Distribution of susceptibility results of isolates of *Staphylococcus aureus* (A) or streptococci (B) to penicillin

6.5 Discussion

Antimicrobial susceptibility testing to ampicillin, cloxacillin, enrofloxacin, oxytetracycline and penicillin was carried out using agar disk-diffusion and broth microdilution methods on 364 *Staph. aureus*, 41 *Strep. dysgalactiae* and 102 *Strep. uberis* isolates from bovine milk. The inter-test categorical agreement varied depending on the antimicrobial/causative organism (*Staph. aureus* or streptococci) combination. This was also true for individual streptococci. An inverse correlation between the sizes of zones of inhibition and MIC levels existed for isolates of *Staph. aureus* to ampicillin, cloxacillin, enrofloxacin and penicillin and for isolates of *Strep. uberis* to ampicillin, cloxacillin, neomycin, oxytetracycline and penicillin. Isolates of *Strep. dysgalactiae* demonstrated no correlation between the results obtained by the two methods.

The overall inter-test categorical agreement overall was 93%. This is similar to findings from another study of isolates of animal origin (Franklin and Wierup 1982). A high level of inter-test categorical agreement was observed to all five antimicrobials for *Staph. aureus* isolates with <5% disagreement. The inter-test categorical agreement for streptococcal isolates was lower and high rates of minor errors were observed to ampicillin, enrofloxacin, oxytetracycline and penicillin. A high rate of major errors was observed for isolates of *Strep. uberis* to cloxacillin, whilst all isolates of *Strep. dysgalactiae* demonstrated inter-test categorical agreement to the same antimicrobial. Agreement in the interpretation criteria for isolates of *Staph. aureus* isolated from milk to penicillin (Myllys *et al.* 1992) and tetracycline (Ferreiro and Biberstein 1978) have been reported previously. However, others have reported various levels of disagreement (Ferreiro and Biberstein 1978; Schlegelova *et al.* 2001; Klement *et al.* 2005).

Disagreement in the inter-test categorical interpretation can be a result of testing error, chance and unknown or incorrectly defined breakpoints. Testing errors may occur due to differences in the type of results obtained and non-standardised testing. The present study was carried out using a standardised methodology (Watts *et al.* 2008) and quality controlled conditions, minimising the occurrence of potential testing errors. However, the MIC values are based on a 2-log distribution, while the sizes of zones of inhibition are continuous measures. Therefore, some discrepancies may occur, particularly around the breakpoint values (Pengov and Ceru 2003). To avoid major errors a margin of at least

3 mm should be allowed as a buffer zone between the ‘S’ and ‘R’ categories when creating interpretive criteria for the results obtained by agar disk-diffusion test (Brunden *et al.* 1992; Ringertz *et al.* 1997). The isolates in this buffer zone will represent the intermediate category (Ringertz *et al.* 1997). This method minimises the risk of falsely reporting susceptibility (Turnidge and Paterson 2007).

The lack of the intermediate category for the agar disk-diffusion method to ampicillin and penicillin for isolates of *Staph. aureus* may explain the major errors observed. However, this cannot be used to explain the other major errors (cloxacillin/*Staph. aureus*; cloxacillin/streptococci and enrofloxacin/streptococci combinations) as there is an intermediate category for the agar disk-diffusion method. The high proportions of minor errors (ampicillin, enrofloxacin, oxytetracycline and penicillin) for streptococci were unlikely to result solely from chance variability. Chance variability should affect all antimicrobial/causative organism combinations equally (Myllys *et al.* 1992; Snell 1994). Moreover, the high level of categorical disagreement between the two tests observed for isolates of streptococci to enrofloxacin cannot be explained purely by the lack of detection of the low intrinsic susceptibility of streptococci to fluoroquinolones. Some of the conflicting results may be due to the use of breakpoints for class representatives of antimicrobials rather than the actual antimicrobial of interest used in the present study. The results obtained from the disk-diffusion and microdilution testing may differ for individual antimicrobials from the same antimicrobial class. This results from differences in the activity of the antimicrobials within the class and the variation in their ability to diffuse in the agar used for testing *in vitro*. However, this discrepancy was not apparent for the oxytetracycline/*Staph. aureus* combination, despite the use of breakpoints set for tetracycline.

The last and most likely explanation for the disagreement in the inter-test categorical interpretation of the results is that the breakpoints for the disk-diffusion antimicrobial susceptibility testing for the mastitis-causing organisms tested in the present study need correction and validation. The disagreement in the inter-test categorical interpretation of results, due to incorrect breakpoints, has been previously reported (Brunden *et al.* 1992; Kibsey *et al.* 1994). A single breakpoint is commonly set for all isolates of a particular causative organism from various clinical conditions. Such a breakpoint is then used to predict the likelihood of the treatment outcome. Moreover, testing susceptibility at a genus level may be misleading as was observed for the streptococci in the present study,

inasmuch as there were discrepancies in the inter-test categorical agreements for *Strep. dysgalactiae* and *Strep. uberis*. Therefore, breakpoints validated for various causative organisms, at a species level, and not for different clinical conditions are likely to increase the proportion of inter-test agreement. Eventual correction and validation of categorical breakpoints could result in enhanced ability of conventional *in vitro* antimicrobial susceptibility testing by agar disk diffusion to predict the outcome of mastitis therapy with sufficient accuracy.

Ideally for optimal estimation of the outcomes of mastitis treatment, the clinical pharmacology of various drugs should also be taken into consideration. The pharmacokinetics of commonly used mastitis treatments is lacking. This is further compounded by the lack of clinical pharmacokinetics of udders with mastitis, as distinct from healthy udders, is scarce (Mercer *et al.* 1974). The lack of data prevents assessment of whether the MICs are achievable and the breakpoints are valid.

Current reporting of the results of agar disk-diffusion testing to practicing veterinarians includes only the S, I and R categories. The inverse correlation between the results of the two tests was in agreement with previous findings (Watson *et al.* 1991). A weak or nil correlation was detected for isolates of *Strep. dysgalactiae*. It can be assumed that larger sample size may allow correlations to become significant. The P-values of the intercepts for all antimicrobial/*Strep. dysgalactiae* combinations were highly significant ($P < 0.001$). This indicates that the sample size was sufficient.

Similarly, the slope and the inverse correlation were weak for isolates of individual causative organisms to neomycin. This may be due to the very narrow range of MIC for *Staph. aureus* (0.012 – 1 µg/mL) and streptococci (mostly between 4 and 64 µg/mL). The same principle can apply to the lack of correlation for isolates of streptococci to enrofloxacin (MIC range 0.25 - 2 µg/mL). However, the narrow range of MIC (0.12 - 2 µg/mL) did not affect the correlation for the isolates of *Staph. aureus* to the same antimicrobial. The narrow range of MIC tends to reduce the correlation coefficient (Baker *et al.* 1991). The discrepancy in the results to neomycin seems to be clinically irrelevant, as mastitis treatment products containing aminoglycosides are now not available in the New Zealand market (Petrovski *et al.* 2011).

Reporting to veterinarians by veterinary diagnostic laboratories in future preferably would include the sizes of zones of inhibition and the current breakpoints as references.

This would allow veterinarians to make more informed decisions on the choice of antimicrobial, based on the size of the zones of inhibition relative to the breakpoints. A sound knowledge of the pharmacodynamics and pharmacokinetics of each antimicrobial is also required. This in turn should result in improved mastitis treatment outcomes.

6.6 Conclusion

The inter-test categorical agreement and inter-test correlation vary depending on the combination of antimicrobial/causative organism at a genus level. Isolates of *Staph. aureus* show better inter-test agreement and correlation than the streptococcal isolates. New breakpoints are required for the antimicrobials used in mastitis treatment for common mastitis-causing organisms. The breakpoints should be at bacterial species level. Reporting of results from agar disk-diffusion tests by veterinary diagnostic laboratories should include the sizes of zones of inhibition and include the breakpoints used for categorical interpretation as a reference.

6.7 Acknowledgments

Financial support from *Bomac Ltd*, Auckland, New Zealand is greatly appreciated. Thanks to Hassan Hussein and Mohamed Abdalla for their help with the testing of the isolates. Alex Grinberg is thanked for his suggestions to improve the quality of the manuscript. KR Petrovski's position at Massey University is supported by *Bomac a company of Bayer Ltd*.

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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Kiro R Petrovski

Name/Title of Principal Supervisor: Prof Norman B Williamson

Name of Published Paper: Correlation of agar disk diffusion and broth microdilution methods when testing the antimicrobial susceptibility of *Staphylococcus aureus* and streptococci isolated from bovine milk samples collected in New Zealand

In which Chapter is the Published Work: Chapter 6

What percentage of the Published Work was contributed by the candidate: 75%

Candidate's Signature

11 July 2011

Date

Principal Supervisor's signature

14/7/2011

Date

Part 2

**Effects of milking frequency on
pharmacokinetics of penicillin G
administered by the intramammary route**

Chapter 7

**Introduction to Part Two: Effects of milking frequency on
pharmacokinetics of penicillin G administered by the
intramammary route**

7. Introduction to Part Two: Effects of milking frequency on pharmacokinetics of penicillin G administered by the intramammary route

In Part Two of this thesis, an *ex vivo* study is reported to address the effects of various milking and treatment regimes on the basic pharmacokinetics of penicillin G in milk after intramammary administration.

The bovine udder is a difficult organ in which to study drug kinetics (Ziv 1980b). Several factors make studies of kinetics of drugs in the udder difficult. These include udder morphology, the accessibility of the duct and alveoli for sampling and differences in the activity of various portions of the gland at different milkings. Therefore, despite the long history of use of antimicrobial compounds to treat intramammary infections in dairy cows, understanding of their pharmacokinetic properties in the mammary gland remains limited. A thorough understanding of the factors that influence drug pharmacokinetics in the mammary gland should contribute to rational intramammary treatment with high therapeutic efficacy and safety for consumers and dairy producers. The most commonly employed procedures are to measure the excretion rate of a particular drug in milk and the proportion of drug recovered from milk (Ziv 1980a, 1980c; Gehring and Smith 2006).

Three major properties of a drug, namely the pharmaceutical, pharmacokinetic and pharmacodynamic behaviours influence the establishment and maintenance of the concentration of the active antimicrobial compound at the site of infection. This is the key pre-requisite for providing contact with the mastitis-causing organisms and, thus, ultimately affecting treatment efficacy.

The rate and quantity of the antimicrobial compound that is released in milk from the formulation depends on the properties of the formulation, vehicle and the compound itself (Mol 1975, Ziv 1980a, 1980c). Manufacturing or formulation modulations (e.g. changes in the salt-form of the antimicrobial compound, solubilising, microencapsulation, gelling or adding a wetting agent) can result in significant changes to the pharmaceutical availability characteristics of a compound from the formulation (Uvarov 1969; Brander 1975a; Gehring and Smith 2006). The use of antimicrobials in the treatment of bovine mastitis has led to an extensive interest in vehicles and

formulations for intramammary administration. The effect of physico-chemical properties of the vehicles on the pharmaceutical availability of the antimicrobial compound were investigated intensively during the 1940s, 1950s and 1960s. An ideal vehicle intended for use in the treatment of mastitis during lactation would be one which affords maximal concentration of the antimicrobial immediately after infusion and obtains the maximum amount of surface contact with the mastitis-causing organisms (Hueber *et al.* 1960). However, this statement ignores the categorisation of antimicrobials into time-dependent and concentration-dependent. This categorisation is nowadays regarded as important for therapeutic outcome after antimicrobial treatment of infectious diseases.

Drug pharmacokinetics deals with the kinetics of absorption, distribution, metabolism and elimination (Ziv 1975). These properties together are generally defined by the mathematical description of the changes in drug concentrations in the body. It is possible to design the dosage schedule based on quantitative data for these properties, particularly when coupled with an understanding of the physiological disposition of the antimicrobial compound levels in the tissues. Drug recovery is affected by numerous factors, namely formulation, vehicle, antimicrobial compound, milk production, absorption, distribution and metabolism of the compound. Thus, simply observing the drug recovery from milk does not allow for estimation of the levels achieved in tissues (Ziv 1975; Toutain *et al.* 2002).

It is recommended that intramammary treatments are given after milking in order to minimise the dilution of the administered dose by milk in the udder. Treatment after milking also allows removal of the inflammatory products that block the milk-duct system. Antimicrobial compounds administered by the intramammary route generally diffuse rapidly to the dorsal portions of the mammary gland (Ullberg *et al.* 1958b; Ehinger and Kietzmann 2000b) provided there are no obstructions or blockage of ducts by inflammatory debris as can occur in acute cases or chronic cases of mastitis (Ullberg *et al.* 1958b; Uvarov 1969).

Some antimicrobial compounds administered by the intramammary route are partially absorbed from the gland into the general circulation (Rasmussen 1964, 1966; Uvarov 1969; Mercer *et al.* 1974a; Mercer *et al.* 1974b; Ziv and Sulman 1975; Ziv 1976) which is the main explanation for the progressive reduction in milk concentrations of drugs

during sequential sampling. Penethamate administered by the intramammary route was absorbed twice as fast as urea and ampicillin, four times as fast as methicillin and eight times as fast as benzyl-penicillin (Ziv and Sulman 1975). Cephalexin was absorbed eight times faster than cephaloridine (Ziv 1975). The speed of absorption of penicillin from the mammary glands was also affected by the vehicle of the formulation. It was absorbed faster from aqueous and colloidal silica formulations than from aluminium monostearate formulations (Mercer *et al.* 1974a). This was probably due to the slower dissolution from the latter formulations.

The physico-chemical properties of a drug determine its tissue penetrating ability, together with the proportion of unbound drug available to combat mastitis-causing organisms. For most antimicrobial compounds, the fraction of the drug that is non-ionised, lipid-soluble and not bound to the serum or milk proteins, is the proportion which penetrates the blood-milk barrier by passive diffusion (Rasmussen 1964; Ziv *et al.* 1974). The validity of this concept has been confirmed for a variety of antimicrobial compounds, including ampicillin, erythromycin, lincomycin, penethamate hydriodide, penicillin G, rifampin, rifamycin, spectinomycin, sulphonamides and trimethoprim (Rasmussen 1964; Ziv *et al.* 1973a; Ziv and Sulman 1973; Ziv *et al.* 1974; Ziv and Sulman 1975; Ziv 1976).

Oily vehicles confer many advantages upon the manufacturing of intramammary products. Chemical stability of many antimicrobial compounds is better in oils (Vigue 1959; Funke 1961) and the process of manufacturing is easier (Schipper 1955; Ullberg *et al.* 1958b, 1958a; Vigue 1959). Oils are not absorbed from the mammary gland (Funke 1961) resulting in an extended period of activity (Funke 1961) and greater concentrations of the active compound in milk and tissue (Schipper 1955; Vigue 1959; Funke 1961; Ehinger and Kietzmann 2000b) allowing for longer inter-treatment intervals. Furthermore, due to their low specific weight it has been postulated that oily suspensions of penicillin carry the antimicrobial up to the dorsal portions of the udder more easily than the aqueous solutions due to their low density (Ullberg *et al.* 1958b). Authors related all of their findings of the differences in the concentrations of the antimicrobial compounds achieved in the various portions of the mammary gland to the physico-chemical properties of the vehicles. They completely ignored the biological principles in the explanation, such as the gland-drug interaction and massage action by movement of the cow.

No difference was found in the distribution of radiolabelled S³⁵-penicillin between the samples of mammary glands one or eight hours after administration as determined by autoradiography after slaughter (Funke 1961). However, the concentrations in milk and tissue were higher in the samples collected one hour after treatment than in later one (Funke 1961). A possible explanation for the latter finding is that time allowed for absorption, hydrolysis or metabolism of the antimicrobial compound to occur. In contrast, the longer the period between drug administration and slaughter, the more uniform was its distribution in the gland (Rasmussen 1964).

The speed of elimination of antimicrobial compounds from the mammary gland, after administration by the intramammary route, is chiefly governed by the formulation, antimicrobial properties, vehicle, dose, treatment regime, biological variation between individual animals, the milk yield of the cow, the health status of the gland and frequency of milking. The formulation and manufacture of intramammary products are highly important in determining elimination times from milk (Uvarov 1960; Mercer *et al.* 1970; Mol 1975; Ziv 1980c; Ehinger and Kietzmann 2000a, 2000b; Gehring and Smith 2006). Some of the factors that have been identified as being of significance include differences in antimicrobial fractions, ratios between antimicrobials and vehicles, particle size, purity of compounds, homogenisation, mixing, drug tissue distribution and chemical interactions of the ingredients (Mol 1975; Ehinger and Kietzmann 2000a, 2000b).

Studies on products containing penicillin administered by the intramammary route have indicated that elimination times from milk, in cows milked twice daily, are between 28 and 144 hours (Uvarov 1960; Edwards 1964; Mercer *et al.* 1970; Ziv *et al.* 1973b; Mol 1975). For example, aqueous solutions of sodium benzyl penicillin G administered to single quarters in dosages up to 100,000 IU had elimination times from milk of 36 - 72 hours (Mol 1975), whilst the addition of waxes and aluminium monostearate resulted in prolonged excretion time of over 72 hours (Mol 1975). On the other hand, Albright *et al.* (1961), Edwards (1964) and Mercer *et al.* (1970) showed that excretion times were independent of the formulation or penicillin concentrations, as long as aluminium stearate (or a derivative) was not added (Albright *et al.* 1961; Edwards 1964; Mercer *et al.* 1970).

In an oily vehicle containing an oil/water emulsion consisting of water plus 4% wax and 5% peanut oil, elimination times from milk were 24 - 48 hours (Mol 1975). Adding

vegetable oils (with or without waxes) prolonged the elimination time to well over 96 hours (Mol 1975). The addition of a Tween detergent to such a vehicle reduced the milk elimination times to 72 - 96 hours (Mol 1975). Interestingly, the addition of aluminium monostearate to the emulsion did not lead to a substantial increase in the elimination times (Mol 1975), although these findings were in contrast to several other reports (Uvarov 1960; Edwards 1964; Mercer *et al.* 1970; Brander 1975b) who suggested that the addition of aluminium monostearate extended the time to elimination.

Oil/water emulsion formulations containing penicillin in vehicles with added paraffin wax have been reported to have elimination times from milk of 4 - 6 days for treated quarters and 0.4 - 0.8 days for untreated quarters (Mol 1975). The presence of penicillin in untreated quarters is most likely to be due to absorption into the general circulation and re-distribution. Theoretically there is the possibility of some local mode of transfer between treated and untreated quarters, although biological principles do not support this notion (Linzell 1971; Knight *et al.* 1994; McManaman and Neville 2003). Penicillin in a vehicle of polysorbatum 65 and 85, peanut oil, castor oil and dodecyl gallate was reported to have elimination times from milk of 2.5 - 3.5 days for treated quarters and 0.5 - 1.5 days for untreated quarters (Mol 1975). Other studies of penicillin in oil-based vehicles have reported milk elimination times of 36 - 144 hours (Uvarov 1960; Mercer *et al.* 1970; Ziv *et al.* 1973b).

The effect of the actual dose of penicillin upon its pharmacokinetics is somewhat controversial. Increasing the concentration of penicillin in the formulation extended milk elimination times (Uvarov 1960; Mol 1975), but it has not been considered as a significant effect in most other reports (Mercer *et al.* 1970; Ziv *et al.* 1973b; Ziv 1980c), at least, providing the dose increase did not exceed 10 times the original (Mol 1975). The findings of Blobel and Burch (1960) for an oxytetracycline-based intramammary formulation were rather different to those of penicillin. They reported that an aqueous solution containing 426 mg of oxytetracycline given as a single treatment was completely eliminated by 60 hours. Doubling the dose (852 mg per quarter) resulted in detectable concentrations for up to 72 hours (Blobel and Burch 1960).

The number of treatments also affects the elimination times from milk. Extended treatment results in a prolonged elimination time from milk (Brander 1975a; Mol 1975; Whitem 1999; Smith *et al.* 2004), particularly when coupled with low milk yields (Brander 1975a; Mol 1975). This is likely due to the accumulation of the antimicrobial

compound in the residual milk, udder tissues and other organ systems, resulting in a continued release and re-distribution in the milk.

Previous studies on milk elimination times from the udder of antimicrobials administered by the intramammary route have indicated the extent of normal biological variation between individual cows (Mercer *et al.* 1970; Ziv *et al.* 1973b; Mol 1975; Knappstein *et al.* 2003). Both milk yield and the physiological status of the infused gland have significant effects on the milk elimination times of antimicrobials (Mercer *et al.* 1970; Mol 1975; Gehring and Smith 2006). Increased milk yield has usually been associated with shorter elimination times (Mercer *et al.* 1970; Mol 1975; Knappstein *et al.* 2003; Smith *et al.* 2004). Mol (1975) also reported lower recovery rates of the total administered dose of drug from cows with lower milk yields. The reason for this finding was not given and is still lacking.

Cows with increased somatic cell levels in milk also display prolonged elimination time of antimicrobials from milk (Mercer *et al.* 1970; Jayachandran *et al.* 1990). Quarters affected by intramammary infections have prolonged elimination time, which is further affected by the severity of inflammation (Edwards 1964; Mercer *et al.* 1970; Mercer *et al.* 1974b; Mol 1975) although this depends upon the drug and its formulation. It may be that such differences in the elimination times from milk between cows with healthy udders, quarters/udders with mastitis or low and high somatic cell count were result of changes in the composition of the milk secretion occurring during intramammary infections, leading to changes in the pharmacokinetic properties of the antimicrobial compound.

Elimination of antimicrobial compounds has been studied numerous times for regulatory purposes. For these, elimination time is investigated in normal animals without mastitis. The relevance of such data to the situation in the intended target is questionable given that intramammary products are intended for administration to quarters/cows with mastitis. Establishment of elimination times from milk in quarters/udders with mastitis has been of interest in research (Mercer *et al.* 1974a; Mercer *et al.* 1974b), but is not used for regulatory purposes because of problems in standardisation of affected quarters. To minimise variability, experimentally-induced mastitis models have been investigated (Mercer *et al.* 1974a; Mercer *et al.* 1974b). Nonetheless, there are concerns regarding this approach, since the standardisation is not absolute nor does experimentally-induced

mastitis represent a natural condition with respect to differences in causative organisms, stage of infection, number of quarters affected, etc.

Milking invariably reduces the concentration of an active compound (Brander 1975a; Mol 1975). Frequent milking shortens elimination times (Mol 1975; Knappstein *et al.* 2003; Stockler *et al.* 2009). Movement to once-a-day milking is currently unique to the New Zealand dairy industry. The effects of this milking frequency on the pharmacokinetic of penicillin G administered by the intramammary route compared to the 'normal' twice daily milking are unknown. Therefore, a study aiming to investigate changes in the pharmacokinetics of various milking frequency and treatment regime was designed and is reported in the Chapter 8 of this thesis.

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Chapter 8

Milking frequency affects the penicillin G elimination times from milk, concentrations and recovery rate following intramammary administration to dairy cows

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8. Milking frequency affects the penicillin G elimination times from milk, concentrations and recovery rate following intramammary administration to dairy cows

8.1 Abstract

AIMS: The effects of milking treated cows twice daily (TD) or once-a-day (OAD) on the patterns of concentrations of penicillin G s in milk, including concentrations achieved in milk, proportion of recovered drug from milk and elimination time (WHP), following administration of an intramammary product containing 1,000,000 IU penicillin G, *Aloe vera* and *Centella* extracts (Lactapen G, *Bomac Laboratories Ltd*, New Zealand) were investigated.

METHODS: Thirty healthy lactating dairy cows were treated at three (3xOAD and 3xTD) or six (6xTD) consecutive milkings. The WHP was defined as the time from the last treatment at which the concentration of penicillin G fell below 0.004 mg/kg. The time above the minimal inhibitory concentrations (MIC) (T>MIC) was calculated as the number of hours when the concentrations of penicillin G were above the MIC₉₀. The amount of penicillin G recovered in milk was estimated as the mass of the recovered penicillin G summed for each milking.

RESULTS: The WHP was 82.7 ± 2.6 , 57.3 ± 2.3 and 63.4 ± 2.1 hours for 3xOAD, 3xTD and 6xTD, respectively. The WHP was significantly longer for OAD than for either TD regime ($P < 0.001$), but the difference between 3xTD and 6xTD was non-significant ($P = 0.053$). The T>MIC for *Streptococcus uberis* and *Staphylococcus aureus* were significantly greater ($P < 0.05$) for 3xOAD (98 ± 4.1 and 63 ± 2.6 hours) and 6xTD (105 ± 4.4 and 77 ± 3.2 hours) than for 3xTD (64 ± 2.6 and 41 ± 1.7 hours). Penicillin G is a time-dependent antibiotic. Hence, its efficacy should be significantly increased from extended treatment (*i.e.* 6xTD) or milking once-a-day. The amount (and proportion) of drug recovered in milk was different between the groups ($P < 0.001$) being $1,398 \pm 249$ (46.6%); $2,665 \pm 249$ (88.8%) and $4,398 \pm 249$ mg (73.0%) for 3xOAD, 3xTD and 6xTD, respectively.

CONCLUSIONS: Penicillin G WHPs and proportion of recovered drug are affected by the milking frequency. Doubling the number of treatments or milking OAD resulted in a significantly increased T>MIC which may achieve higher cure rates. Longer retention times of penicillin G into the udder in OAD increases the likelihood of absorption into the general circulation and/or local degradation.

KEY WORDS: penicillin G, udder, milking frequency, time above the MIC, withholding period

MIC – Minimal Inhibitory Concentrations, MRL – Maximum Residues Limit, OAD – Once-a-Day, TD – twice daily, WHP – Withholding Period

8.2 Introduction

Intramammary administration of some antimicrobial drugs and courses is a convenient method for treating bovine mastitis. Drugs administered by that route achieve a high antimicrobial concentration at the site of infection without significant systemic absorption (Ziv 1980; Gruet *et al.* 2001; Gehring and Smith 2006). Therefore, intramammary administration limits unwanted systemic side effects, particularly exposure of normal micro flora resident in other body systems, and reduces the risk of tissue residues by reducing the total dose administered. Avoiding unnecessary exposure to antimicrobials decreases the risk of development of antimicrobial resistance. In milk-producing animals it is important that concentrations of antimicrobials reduce to safe levels before milk is harvested for human consumption. The European Agency for the Evaluation of Medicinal Products established the Maximum Residue Limit (MRL) for penicillin G in milk of cows at 0.004 mg/kg (Anonymous 2005).

Bacteriological cure rates following bovine mastitis treatments vary widely. Studies undertaken in New Zealand have reported these to be between 45% and 90% (McDougall 1998; McDougall *et al.* 2007; Bryan and Emslie 2010a). The success of treatment for bovine mastitis is influenced by the duration of treatment, such that extended treatments are reported to achieve better cure rates (Gillespie *et al.* 2002; Hillerton and Kleim 2002; Oliver *et al.* 2003; Oliver *et al.* 2004). The likely success of treatments following administration of antimicrobials may be indicated using a pharmacokinetic/pharmacodynamic approach (Craig 1995; Toutain 2003b; Drusano 2004).

Most dairy cows in New Zealand are milked twice daily. However, some farms milk cows once-a-day throughout the season. Under certain circumstances some herds that normally milk twice daily switch the whole or a part of the herd to once-a-day milking. This can occur in early lactation, through the mating period, in late lactation and before drying-off. This is a common procedure for cows that are in low body condition score, are young or are at-risk-cows (*i.e.* cows that had a difficult calving, twins, retained fetal membranes and have been ‘downer cows’). Milking once-a-day is also commonly practiced in cows with mastitis during their treatment and the withholding period, particularly in large herds. This is in contrast to historical practice to strip infected quarters/cows for six to eight times per day. The effects of milking once-a-day on the concentrations of penicillin achieved in the milk of treated cows, amounts of recovered drug and elimination times are unknown.

The objectives of this study were to determine the elimination times from milk of an intramammary mastitis product containing penicillin G in cows milked at normal or reduced milking frequencies. Additionally, the persistence of an effective concentration of penicillin G, and the amount of drug recovered in milk were estimated.

8.3 Materials and methods

Experiment designs were approved by Grassland Animal Ethics Committee (GAEC 10891) and Massey University Animal Ethics Committee (MUAEC 10/53).

8.3.1 Experimental animals

Fifty-four healthy lactating cows with 4 functional quarters, no signs of intramammary infection, producing more than 10 L of milk daily and no antimicrobial treatments within 30 days of study commencement were used in two experiments. The first experiment was conducted in cows milked twice daily (TD) at the *Agricultural Farm Services*, Massey University Number 4 dairy herd. They were grazed at pasture and supplemented as necessary with silage and Palm Kernel Extract to maintain body condition and production as required. Twenty cows were treated at three consecutive milkings (3xTD) and 20 cows were treated at six consecutive milkings (6xTD). The second experiment was conducted in cows milked once-a-day, on a different commercial dairy farm, with 10 cows treated on three consecutive milkings at 24-hourly intervals (3xOAD). On this farm cows were grazed at pasture, supplemented with Palm Kernel Extract and molasses as

necessary to maintain body condition and production. In each experiment two further cows remained untreated as negative quality controls.

Cows were ranked according to milk production (4-day average preceding the day of allocation). Equal numbers of cows with low, medium and high average milk yields were assigned to each group.

Cows were grazed at pasture and milked at approximately 6:00 and 16:00 daily if milked twice daily or at 24-hourly intervals if milked once-a-day approximately at 8:00.

The following records were available for each cow: cow id, breed, parity, days-in-milk, pre-treatment milk yield, somatic cell count, fat and protein.

8.3.2 Treatment and procedures

Antibiotic was administered to cows after completion of machine milking by an intramammary infusion using a partial insertion technique (Boddie and Nickerson 1986) to all four quarters (Figure 8.2). The antibiotic formulation contained 1,000,000 IU of penicillin G, and *Aloe vera* and *Centella* extracts in a fast release oily base (Lactapen G; *Bomac Laboratories Ltd*, Auckland, New Zealand).

Milk samples (Figure 8.2) were collected using in-line sampling ports (*TruTest*, Hamilton New Zealand or *DeLaval*, Belgium; Figure 8.3) from all cows starting with the milking before the first treatment until approximately 120 hours after the last treatment. This sampling method ensured that representative samples of the total milk were obtained. The in-line sampling port continuously takes a fixed fraction of the milk harvested throughout milking, assuring that the sample is representative of all of the milk for that milking.



Figure 8.1. Treatment by the intramammary route using partial insertion technique

Milk specimens were screened qualitatively for inhibitory substances using a Copan Milk Test (*Copan Diagnostics Inc*, Corona, USA) at SAITL Dairy Laboratories, Hamilton, New Zealand. Milk composition and somatic cell counts were tested using a Combifoss (*Foss*, Hilleroed, Denmark) and Fossomatic 5000 (*Foss*) instruments. A sample from each collection time after the last treatment was tested until two consecutive negative results were obtained. Positive samples were tested quantitatively in serial dilution using the Copan Milk Test and BSDA plates (*Fort Richard Laboratories Ltd*, Auckland, New Zealand) in parallel.

8.3.3 Statistical analysis

Concentrations of penicillin G in milk were \log_{10} -transformed and regressed on time after the last application. Using the regression line for each cow, milk elimination time in independent analysis for each treatment regime and milking frequency was predicted as the time at which the projected concentration of the drug fell below 0.004 mg/kg (Vranic *et al.* 2003), using the GLIMMIX procedure of SAS (Statistical Analysis System, *SAS Institute Inc.*, Cary, NC, USA) version 9.2. Analyses of variance were carried out to

identify factors that influenced the elimination times of penicillin G from milk using the GLIMMIX procedure. The model considered the fixed effects of individual cow, cow parity, days-in-milk, pre-treatment somatic cell count and pre-treatment daily yields of milk, fat and protein. However, none of these factors, except the effect of individual cow, had a significant effect on the elimination times of penicillin G from milk. Therefore, the final model included only the effect of an individual cow.

The time above the minimal inhibitory concentrations (MIC) was calculated in number of hours when the concentrations of penicillin were above the published MIC₉₀ of 4 and 0.06 mg/kg for *Staphylococcus aureus* and *Streptococcus uberis*, respectively (Salmon *et al.* 1998).

The amount of penicillin G recovered in milk was estimated as the sum of the penicillin G measured at each milking, where the penicillin G at each milking was calculated from the volume of milk at that milking times the concentration of penicillin G in that milk. For each cow, the total amount of penicillin G recovered in milk, the proportion of drug recovered (*i.e.* estimate of drug recovered divided by total amount of drug administered) and the average amount of penicillin G recovered per milking were calculated. Analyses of variance to identify factors that influenced the amount of penicillin G recovered in the milk of treated cows were carried out using the GLIMMIX procedure of SAS. The model considered the fixed effects of parity, days-in-milk, pre-treatment somatic cell count and pre-treatment daily yields of milk, fat and protein. However, none of these factors had a significant effect ($P>0.05$) on the amount of penicillin G recovered in the milk of treated cows. Therefore, the final model did not include them.

The level of significance was set at $P<0.05$.



Figure 8.2. Milk samples for various analysis and reserves



Figure 8.3. *DeLaval* in-line samplers

8.4 Results

8.4.1 Elimination times

Milk elimination times for penicillin G in milk from treated cows are presented in Table 8.1. Each individual cow due to intrinsic biological variability influenced the milk elimination time by an average of -0.35 hours (P=0.026).

Doubling the number of treatments from 3 to 6 in cows milked twice daily resulted in a change in the milk elimination time (3xTD: 57.3 ± 2.3 , 6xTD: 63.4 ± 2.1 hours), although the difference just failed to reach statistical significance (P=0.053). However, the milking frequency significantly affected the elimination times from milk. Cows treated 3 times and milked once-a-day (3xOAD) had longer elimination times of penicillin from milk (82.7 ± 2.6 hours) compared with cows in the 3xTD group (57.3 ± 2.3 hours; P<0.001).

Table 8.1. Elimination times of penicillin G from milk (means \pm SE) in cows under different milking frequency and treatment regime treated with Lactapen G by the intramammary route

Treatment group	Elimination times (hours)
3x OAD ¹	82.7 ± 2.6^a
3x TD ²	57.3 ± 2.3
6x TD ³	63.4 ± 2.1

¹Cows treated three times at 24-hourly intervals and milked once-a-day; ²Cows treated three times at 12-hourly intervals and milked twice daily; ³Cows treated six times at 12-hourly intervals and milked twice daily; ^aValues within columns with different superscripts differ (P<0.05).

8.4.2 Time above MIC

The time above the MIC was affected by the treatment and milking frequency. Estimated times above the MIC for *Strep. uberis* and *Staph. aureus* were significantly (P<0.05) greater for 3xOAD (98 ± 4.1 and 63 ± 2.6 hours) and 6xTD (105 ± 4.4 and 77 ± 3.2 hours) than for 3xTD (64 ± 2.6 and 41 ± 1.7 hours). These data are illustrated in Figure 8.4.

8.4.3 Amount of drug recovered

The amount of penicillin G recovered from milk varied (overall difference in the recovered amount $P < 0.005$; Table 8.2). A higher proportion of recovered drug was observed in cows milked twice daily (3xTD and 6xTD) than in those milked once-a-day (3xOAD; $\geq 73\%$ vs. 47%, $P < 0.001$). Furthermore, the average amount of drug recovered per milking was > 20 mg for 3xTD and 6xTD cows, compared to 9 mg per milking for 3xOAD cows once-a-day ($P < 0.001$).

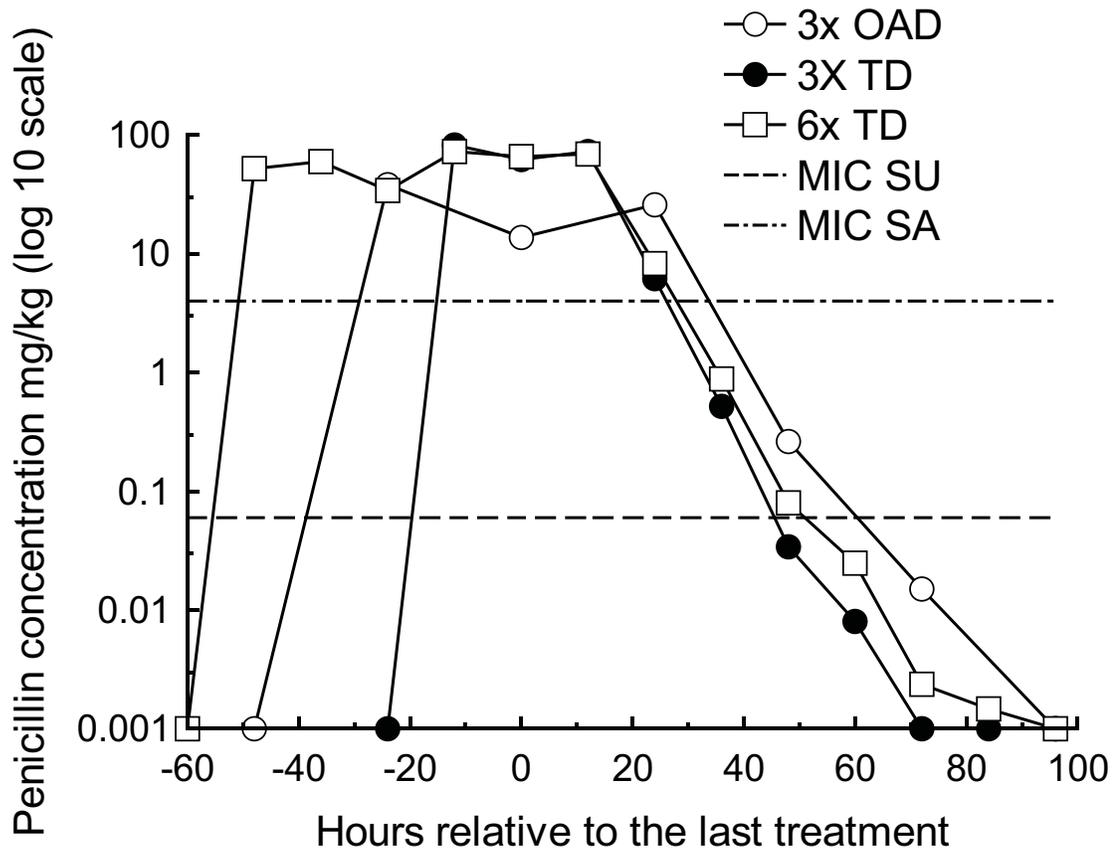


Figure 8.4. Concentrations of procaine penicillin G in milk (mg/kg) in cows treated 3 times and milked once-a-day (-o-) or twice daily (-●-) and treated 6 times and milked twice daily (-□-). MIC₉₀ for *Streptococcus uberis* (- - -); MIC₉₀ for *Staphylococcus aureus* (- · -). MIC values from Salmon *et al.* (1998)

Table 8.2. Amount of penicillin G recovered from milk of cows under different milking frequency and treatment regime treated with Lactapen G by the intramammary route

Treatment group	Total (mg)	Percentage recovered ¹	Average concentration (mg/kg) ²
3x OAD ³	1,397.8 ± 248.8 ^{ab}	46.6	8.7 ± 1.7 ^{ab}
3x TD ⁴	2,664.7 ± 248.8 ^{ac}	88.8	20.4 ± 1.7 ^{ac}
6x TD ⁵	4,379.7 ± 248.8 ^{bc}	73.0	28.0 ± 1.7 ^{bc}

¹Percent of the total drug administered; ²Average concentration of drug per kg milk ; ³Cows treated three times at 24-hourly intervals and milked once-a-day; ⁴Cows treated three times at 12-hourly intervals and milked twice daily; ⁵Cows treated six times at 12-hourly intervals and milked twice daily; ^{a-c}Values within columns with different superscripts differ (P<0.05).

8.5 Discussion

This investigation of the concentrations of penicillin G achieved in milk after intramammary infusion, amounts of drug recovered and elimination times from milk in lactating cattle under different milking frequency is the first study of its kind reported from New Zealand. The time to elimination of penicillin G varied between the milking frequencies. In cows milked twice daily, doubling the number of treatments resulted in a significantly increased time above the MIC, which may result in increased cure rates. Extended treatment of cows milked twice daily (6xTD) produced penicillin G concentrations that remained above the MIC for a similar period of time to that in cows treated and milked once-a-day with three treatments (3xOAD). The amount and percentage of drug recovered from milk varied between treatments and milking frequency. Milk from cows treated and milked once-a-day had lower recovery rates than either group milked twice a day. This could be the result of greater systemic absorption from the udder due to the longer period in the udder.

A limitation of the present study is that there were two independent farms where the experiments were conducted. It is a rare situation to have a dual milking frequency regime on a single farm and such a farm was not available for the researchers at the time of the experiments. Therefore, two farms with different milking frequency were used. A second limitation is the estimates of the elimination times of any antimicrobial from milk and achieved concentrations can be affected by assay sensitivity. Instrumental analysis

would have been preferable, but, microbiological assays have been used previously for reports of a similar character (Uvarov 1960; Edwards 1964; Mercer *et al.* 1970; Ziv *et al.* 1973). The sensitivity of the assays used in previous studies reporting elimination times of penicillin from milk ranged between 0.001 and 0.01 mg/kg with the majority being around 0.001 mg/kg. The sensitivity of the assay used in the present study was approximately 0.001 mg/kg, which is less than half of the current MRL (Anonymous 2005). Thus, comparison of the results with previous reports should be valid.

Previous studies on products administered to the udder that contain penicillin indicated elimination times from milk of between 28 and 144 hours (Uvarov 1960; Edwards 1964; Mercer *et al.* 1970; Ziv *et al.* 1973). The elimination times from milk of 57, 63 and 83 hours for all three treatment regimes in the present study fell therefore within the range from previous reports.

Numerous factors can affect the concentrations of antimicrobials and time for their elimination from milk in the bovine mammary gland. A thorough understanding of these factors should contribute to rational intramammary treatment, with high therapeutic efficacy and safety for consumers and dairy producers. The factors that most alter the concentrations achieved in milk and elimination from milk are those associated with the pharmaceutical characteristics of the product or with the treated cow (Ziv 1980; Gehring and Smith 2006).

A challenge for the pharmaceutical industry is to design products that achieve rapid and widespread distribution in the udder, penetration throughout the mammary tissue and rapid elimination from milk. The formulation and manufacturing of intramammary products is therefore of great importance in determining elimination times from milk (Uvarov 1960; Mercer *et al.* 1970; Mol 1975; Ehinger and Kietzmann 2000a). Variations result from differences in antibiotic fractions, ratios between antibiotics and vehicles, particle size, purity of compounds, homogenisation, mixing, drug tissue distribution, type of vehicle and interaction (Mol 1975; Ehinger and Kietzmann 2000a, 2000b). The differences in elimination times of penicillin from milk between the treatment groups observed in the present study cannot be explained by pharmaceutical characteristics as one product was used. Additionally, all cows were treated in each quarter at each scheduled treatment. Therefore, the differences in times to elimination of antibiotic from milk in the present study cannot be explained on the basis of different amounts of

penicillin G administered to cows. Furthermore, cows receiving extended therapy (6xTD) at 12-hourly intervals demonstrated similar elimination time as those treated under a shorter (3xTD) treatment regime. Previously, repeated treatments and low production have been shown to result in a significant extension of milk elimination times (Brander 1975; Mol 1975). However, extended treatment regimes for bovine mastitis treatment generally necessitates an 'off-label' use of the drugs, so establishing withholding periods (WHPs) for either extended use or use in cows that are milked once-a-day would allow these data to be added to the product's label. Interestingly, doubling the number of treatments from 3 to 6 in cows milked twice daily resulted in a non-significant change of less than 7 hours in the milk elimination times. This finding contrasts to the previous report of Mol (1975) and studies conducted on other antimicrobials (Whittem 1999; Smith *et al.* 2004). Possible explanations for this difference are that the formulation of the antimicrobial used in the present study prevents the accumulation of procaine penicillin G in the mammary gland, or perhaps that *Aloe vera* and *Centella* extracts facilitated the elimination of the active ingredient from the mammary gland.

Milking invariably reduces the concentration of an active compound in the udder (Brander 1975; Mol 1975). In the present study, frequent milking reduced the elimination times to an extent that was in agreement with previous reports (Mol 1975; Knappstein *et al.* 2003; Stockler *et al.* 2009b). A portion of the formulations that are administered by the intramammary route remains in the cistern and duct system. Milking flushes part of this portion from the mammary gland. The remaining portion of infused formulation becomes diluted as the gland refills, and the dilution of the active compound continues in a process that proceeds in turn after each milking. Additionally, there is a portion of the antimicrobial compound that is retained in the mammary gland bound to tissues and in the residual milk. Some of the antimicrobial in the milk at the next milking will originate from this portion. Furthermore, re-distribution to the mammary gland of the absorbed portion of the antimicrobial compound occurs. With each milking, the portion of remaining formulation reduces, thus the concentration of the active compound decreases (Mol 1975; Gehring and Smith 2006). Therefore, the duration of the effective concentration of antimicrobial compound and the speed of elimination from milk are affected by the frequency of milkings.

Previous studies on the elimination times of antimicrobials administered by the intramammary route from milk, indicated normal biological variation between individual

cows (Blobel 1960; Mercer *et al.* 1970; Ziv *et al.* 1973; Knappstein *et al.* 2003). The present study emphasises the importance of biological variation, since the cow effect on milk elimination time average -0.35 hours ($P=0.026$). This could not be explained by factors such as age of the cow, stage of lactation, milk, fat or protein yields. Therefore, due to the large variability in elimination times between cows, studies investigating pharmacokinetics of drugs administered by the intramammary route should have enough power to represent the external population. This will prevent incidence of violative residues when the product is used on dairy farms.

Previous studies have demonstrated that milk yield and the physiological status of gland have significant effects on the milk elimination times of intramammary antimicrobials (Mercer *et al.* 1970; Mol 1975; Gehring and Smith 2006). Increased milk yield has usually been associated with shortened elimination times (Mercer *et al.* 1970; Knappstein *et al.* 2003; Smith *et al.* 2004; Smith *et al.* 2009). This was not the case in the present study ($P=0.234$), which is an interesting finding that requires further investigation. Furthermore, the effects of pre-treatment somatic cell level in the present study was non-significant ($P=0.384$; data not shown). This may be a result of the selection criteria since all cows were healthy with no evidence of intramammary infection. Further research is required on the effects of clinical and subclinical mastitis on the pharmacokinetics of penicillin G following intramammary administration.

Beta-lactam antimicrobials, including penicillin, are classified as time-dependent. Their efficacy is proportional to the time their concentration exceeds the MIC of a particular pathogen (Craig 1998; Burgess 1999; Toutain 2003a). In the present study using Lactapen G, extended treatment of quarters or milking cows once-a-day resulted in significantly increased time above the MIC. Thus, these approaches should result in superior cure rates. This pharmacokinetic/pharmacodynamic hypothesis has already been confirmed with the product of interest in cows milked twice daily (Bryan and Emslie 2010a, 2010b).

The amount and proportion of drug recovered from milk varied between the treatment groups. Interestingly, cows treated and milked once-a-day had a smaller recovery. Longer retention times of penicillin G in the udder may have resulted in a higher absorption into the general circulation and/or local degradation of the antimicrobial. This finding indicates that the pharmacokinetics of drugs administered by the intramammary route

can be affected by varying the treatment and milking frequency, as reported previously (Knappstein *et al.* 2003; Stockler *et al.* 2009a, 2009b).

8.6 Conclusion

Milk elimination times and recovery of penicillin G after intramammary treatment differed significantly between cows that were milked once or twice a day. Therefore, it is imperative that each new mastitis formulation is carefully tested in experiments using cows milked at various milking frequencies. Doubling the number of treatments or milking cows once-a-day resulted in a significantly increased time of persistence of effective concentrations in the milk. Thus, it is expected that these management strategies will result in higher cure rates. Longer retention times of penicillin G in the udder of cows milked once-a-day is likely to have resulted in a higher absorption into the general circulation and/or local degradation of the antimicrobial.

8.7 Acknowledgments

This study was financially supported by *Bomac Laboratories Ltd*, Auckland, New Zealand. Staff at *Invoco – AgResearch*, Palmerston North, New Zealand, Mr Jeremy Lind and farms' staff are thanked for their involvement in the animal phase. The staff at *SAITL Dairy Laboratories*, Hamilton, New Zealand, are also thanked for their contribution in the analytical phase. KR Petrovski's position at Massey University is financially supported by *Bomac a company of Bayer Ltd*.

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STATEMENT OF CONTRIBUTION
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(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

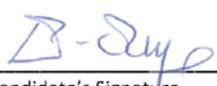
Name of Candidate: Kiro R Petrovski

Name/Title of Principal Supervisor: Prof Norman B Williamson

Name of Published Paper: Milking frequency affects the penicillin G elimination times from milk, concentrations and recovery rate following intramammary administration to dairy cows

In which Chapter is the Published Work: Chapter 8

What percentage of the Published Work was contributed by the candidate: 80%


Candidate's Signature

11 July 2011
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Principal Supervisor's signature

14/7/2011
Date

Part 3

Treatment of heifers pre-calving

Chapter 9

Introduction to Part Three: treatment of heifers for mastitis pre-calving

9. Introduction to Part Three: treatment of heifers for mastitis pre-calving

Part Three of the thesis deals with the importance of mastitis in heifers (2-year-old primiparous cattle). The decision to deal with mastitis in heifers reflects the growing importance of mastitis in these animals. Historically, heifers have been regarded as free of intramammary infections, yet, this assumption has been seriously challenged during the last two to three decades (Tables 9.1 and 9.2).

Replacement animals, particularly heifers, are critical for achieving fast genetic improvement in a herd. They also represent a substantial investment. The goal of operations rearing replacements should be to provide an environment that supports heifers to express their full lactation potential at a desired age and minimal expense. Animal health expenditure has a vital role in heifers achieving their full genetic potential. Various disorders at this stage of life of a heifer, including intramammary infections, have potential to influence the future productivity of heifer replacements. During rearing of heifer-calves and mated heifers they are not observed as frequently as the milking herd and udder health is often overlooked. Such neglect may have long-standing economic implications when an intramammary infection occurs and causes permanent damage to the glandular tissue (Boddie *et al.* 1987; Trinidad *et al.* 1990c; Nickerson *et al.* 1995).

Many pre-partum intramammary infections self-cure rapidly after calving (Oliver and Mitchell 1983; Nickerson *et al.* 1995). This is particularly reported for coagulase negative staphylococci (CNS) (Oliver and Mitchell 1983; Oliver and Jayarao 1997; Compton *et al.* 2007; Piepers *et al.* 2010). However, some heifers remain infected for long periods (Coffey *et al.* 1986; Oliver *et al.* 1992; Roberson *et al.* 1994; Nickerson *et al.* 1995; Aarestrup and Jensen 1997; Calvinho *et al.* 2007). Therefore, it is essential to ensure that any intramammary infections existing in the pre-parturient period are resolved before calving, as these are likely to impact on the future udder health and productivity of the heifer (Compton *et al.* 2007; Piepers *et al.* 2010).

Table 9.1. Reported prevalence of intramammary infections in heifers before the first calving based on culture

Study	Country	Number sampled	Infection rate	Prevalence from the total number of sampled			
				Coagulase-negative staphylococci	Coagulase-positive staphylococci	Streptococci	Coliforms
Heifer level							
da Costa <i>et al.</i> (1996)	Brazil	120	81.6	73.5	4.0	15.8	4.2
Roy <i>et al.</i> (2007)	Canada	428	39.0	-	-	-	-
Roy <i>et al.</i> (2009)	Canada	398	69.0	-	-	-	-
Rad (2007)	Iran	168	35.0	29.7	0.0	1.3	0.7
Compton <i>et al.</i> (2007)	NZ	708	38.0	-	-	-	-
Santos <i>et al.</i> (2004)	USA	280	81.0	-	-	-	-
Borm <i>et al.</i> (2006)	USA and Canada	561	63.0	47.1	-	-	-
Quarter level							
Calvinho <i>et al.</i> (2007)	Argentina	560q	32.1	22.2	4.1	2.8	-
Roy <i>et al.</i> (2009)	Canada	1679q	33.0	26.0	3.0	1.0	-
Krömker and Friedrich (2009)	Germany	336q ¹	79.0	61.0	4.0	6.0	4.0
Nagahata <i>et al.</i> (2006)	Japan	58 q	60.3	37.8	0.0	13.8	0.0
Compton <i>et al.</i> (2007)	NZ	2832 q	22.0	13.5	0.4	2.9	0.2
Parker <i>et al.</i> (2007a)	NZ	503q	15.5	11.9	0.8	2.2	-
Parker <i>et al.</i> (2008)	NZ	4097	18.6	14.4	0.4	3.0	0.3
Fox <i>et al.</i> (1995)	USA	2435q	34.4	27.1	2.9	-	-
Matthews <i>et al.</i> (1992)	USA	144q ²	-	39.0	6.9	-	-
Middleton <i>et al.</i> (2005)	USA	760q	41.8	32.5	3.4	2.8	0.1
Oliver (1987)	USA	300q	22.0	14.7	1.0	3.0	2.3
Oliver and Mitchell (1983)	USA	252q	29.0	22.2	1.2	4.8	4.8
Owens and Ray (1996)	USA	168q	58.3	31.5	14.3	11.9	-
Oliver <i>et al.</i> (1992)	USA	460q	60.7	52.8	1.7	4.3	-
Oliver <i>et al.</i> (1997)	USA	4929q	55.3	-	-	-	-
Oliver <i>et al.</i> (1997)	USA	162q	72.3	55.1	3.2	5.7	-
Oliver <i>et al.</i> (2003)	USA	164q	76.0	65.1	1.8	6.0	3.0
Oliver <i>et al.</i> (2003)	USA	168q	72.0	51.5	5.2	3.7	3.0
Oliver <i>et al.</i> (2004)	USA	199q	60.1	8.0	10.6	3.0	-
Trinidad <i>et al.</i> (1990b)	USA	370q	74.6	46.8	44.2	3.7	-
Borm <i>et al.</i> (2005)	USA and Canada	2232q	53.0	-	-	-	-

¹three times sampled before calving. Results from the last sampling being -30 to 0 days before calving; ²teat orifice swabs

Table 9.2. Prevalence of intramammary infections in heifers around the first calving. Day 0 relative to calving is the day of calving. Where there was some control measure the results show only the untreated group of heifers

Study	Country	Number sampled	Days relative to calving	Infection rate	Coagulase-negative staphylococci	Coagulase-positive staphylococci	Streptococci	Coliforms
Heifer level								
Piepers <i>et al.</i> (2010)	Belgium	244	0-4	80.3	56.6	7.0	11.9	-
Kalmus <i>et al.</i> (2007)	Estonia	68	0	75.0	8.8	1.5	23.5	26.5
Edinger <i>et al.</i> (2000)	Germany	1389	0	52.3	18.6	15.3	4.4	1.8
Rad (2007)	Iran	168	5-15	47.1	36.2	3.2	6.3	0.5
Compton <i>et al.</i> (2007)	NZ	666	Within 5 days	49.0	-	-	-	-
Parker <i>et al.</i> (2008)	NZ	1036	0-5	24.7	13.0	0.7	10.5	0.3
Kirk <i>et al.</i> (1996)	USA	339	0-17	71.0	39.0	-	11.0	16.0
Santos <i>et al.</i> (2004)	USA	280	0	66.4	-	-	-	-
Quarter level								
Calvinho <i>et al.</i> (2007)	Argentina	560q	0-7	17.0	9.1	3.7	1.0	-
Piepers <i>et al.</i> (2010)	Belgium	762q	0-4	48.7	35.3	3.5	5.0	-
Meaney (1981)	Ireland	500q	0	17.0	-	3.2	6.0	3.2
Compton <i>et al.</i> (2007)	NZ	2664q	within 5 days	21.5	9.7	0.6	10.4	0.5
McDougall <i>et al.</i> (2008)	NZ	1496q	0-5	20.1	11.5	0.1	4.9	0.0
Pankey <i>et al.</i> (1996)	NZ	458q	0-4	31.7	21.8	0.9	12.2	0.7
Parker <i>et al.</i> (2007a)	NZ	252q	0-4	12.3	5.2	1.6	7.1	0.0
Parker <i>et al.</i> (2007b)	NZ	252q	0-4	12.3	5.2	1.6	5.6	0.0
Sampimon <i>et al.</i> (2009)	The Netherlands	740q	0	58.4	39.2	4.6	4.1	1.5
Fox <i>et al.</i> (1995)	USA	3168q	0	36.0	21.8	2.8	-	-
Oliver (1987)	USA	299q	0	28.1	14.4	0.7	7.4	4.3
Oliver (1987)	USA	299q	7	18.7	7.0	0.7	6.0	3.7
Oliver (1987)	USA	299q	14	10.3	5.7	0.3	1.3	2.0
Oliver and Mitchell (1983)	USA	128q	0	21.2	18.8	0.8	7.8	4.7
Oliver <i>et al.</i> (1992)	USA	164q	3 and 10	44.5	39.0	0.6	4.9	-
Oliver <i>et al.</i> (1997)	USA	162q	3	60.5	48.8	1.9	7.4	-
Oliver <i>et al.</i> (2003)	USA	164q	3	47.0	37.2	0.0	8.7	0.6
Oliver <i>et al.</i> (2003)	USA	168q	3	63.0	53.4	1.7	5.0	0.8
Pankey <i>et al.</i> (1991)	USA	1533q	0	18.6	11.4	0.7	2.6	2.2

Intramammary infections and teat apex colonisation have been reported in very young stock at 6 to 9 months of age (Boddie *et al.* 1987; Trinidad *et al.* 1990b; De Vliegher *et al.* 2003) and even as early as in 1 day old heifer-calves (White *et al.* 1989). These intramammary infections may persist for more than a year (Boddie *et al.* 1987). The colonisation of the teat apex and keratin may serve as a reservoir for subsequent intramammary infections and mastitis. In New Zealand, the most likely cause of clinical mastitis in heifers is *Streptococcus uberis*. This bacterium does not normally colonise the skin. Intramammary infections in pre-calving heifers are usually subclinical (Daniel *et al.* 1986; Waage *et al.* 1998). However, many of the intramammary infections present before calving persist after calving (Compton *et al.* 2007; Krömker and Friedrich 2009) and become chronic (Trinidad *et al.* 1990c; Oliver *et al.* 2003; Oliver *et al.* 2004).

Rates of clinical mastitis in heifers that have been previously reported are presented in Table 9.3. Some studies have shown that the incidence of clinical mastitis in the peripartum period is higher for heifers than cows (Hogan *et al.* 1989; Barkema *et al.* 1998; McDougall 1999; McDougall *et al.* 2007; Parker *et al.* 2007a). In contrast, Sargeant *et al.* (1998) reported the lowest incidence of clinical mastitis in heifers and a steady rise in older cows with increasing lactation number. Additionally, Petrovski *et al.* (2009) showed that, in Northland region of New Zealand, the highest incidence of clinical mastitis is in mature cows aged over 6 years, closely followed by heifers, and the lowest prevalence in 3 and 4 year-olds. Moreover, there is an increase in the prevalence of clinical mastitis in heifers over time between 1940s and present time (Myllys and Rautala 1995) not followed by the similar trend in mature cows. This has been detected as early as 1942 (Van Rensburg 1942). The prevalence of clinical mastitis caused by contagious mastitis-causing organisms in heifers is lower than in mature cows (McDougall *et al.* 2007; Persson Waller *et al.* 2009; Petrovski *et al.* 2009). The reported prevalence of mastitis-causing organisms associated with clinical mastitis in heifers suggests that most important are coagulase-negative staphylococci, environmental streptococci, coagulase-positive staphylococci and coliforms (Table 9.3).

Table 9.3. Rate of clinical mastitis in heifers around calving or during the first lactation. Day 0 relative to calving is the day of calving. Where there was some control measure the results include only the untreated group of heifers

Study	Country	Number sampled	Days relative to calving	Rate of clinical mastitis	Percent of isolations from clinical cases			
					Coagulase-negative staphylococci	Coagulase-positive staphylococci	Streptococci	Coliforms
Piepers <i>et al.</i> (2010)	Belgium	191	0 to 285	11.5	-	-	-	-
Sargeant <i>et al.</i> (1998)	Canada	961	305	19.8	-	-	-	-
Kalmus <i>et al.</i> (2007)	Estonia	1063	0	6.4	8.8	1.5	30.8	29.4
Myllys and Rautala (1995)	Finland	419,069	-7 to +7	HF ¹ 5.6 AY ² 3.9	-	-	-	-
Edinger <i>et al.</i> (1999)	Germany	1389	7	38.0	-	-	-	-
Edinger <i>et al.</i> (2000)	Germany	149	5	34.2	-	-	-	-
Nordhaug <i>et al.</i> (1994)	Germany	200 q ³	First lactation	20.0	-	-	-	-
Waage <i>et al.</i> (1999)	Norway	1349 q	Pre partum to +14	-	12.8	44.3	21.3	6.7
Compton <i>et al.</i> (2007)	NZ	2784 q	0 - 14	7.0	7.7	2.6	67.5	3.6
Compton <i>et al.</i> (2007)	NZ	696	0 - 14	23.4	-	-	-	-
Laven and Lawrence (2008)	NZ	102	0 to ~78	17.6	-	-	-	-
Pankey <i>et al.</i> (1996)	NZ	428	0 - 5	8.1	10.8	-	67.6	2.7
Parker <i>et al.</i> (2007a)	NZ	252 q	-3 to 14	6.7	23.5	5.9	52.9	0.0
Parker <i>et al.</i> (2007b)	NZ	250 h ⁴	0 - 120	13.6	-	-	-	-
Parker <i>et al.</i> (2008)	NZ	1067	0 - 14	6.7	1.0	0.2	4.1	0.3
Oliver <i>et al.</i> (2004)	USA	164q	Pre-partum	-	65.1	1.8	6.0	3.0

¹Holstein Friesian breed; ²Ayrshire breed; ³q – information at quarter level; ⁴h – information at herd level

The prevalence of intramammary infection before or at calving, high individual cow somatic cell count after calving and/or clinical mastitis in heifers before or after calving have been reported to result in a lower production during the first lactation (De Vliegher *et al.* 2005; Piepers *et al.* 2010), long-term decreased production (Woolford *et al.* 1983; Woolford 1985), a higher level of somatic cell count in the first lactation (Trinidad *et al.* 1990a; Hallberg *et al.* 1995), increased risk of clinical mastitis in the following lactation and increased risk of premature removal from the herd (Myllys and Rautala 1995; Rupp *et al.* 2000; Rupp and Boichard 2000; Compton *et al.* 2007; Piepers *et al.* 2010). Occasionally, heifers with pre-existing intramammary infections calve with one or more non-functional quarters (Trinidad *et al.* 1990c; Nickerson *et al.* 1995) or ones that become non-functional during the first lactation (Compton *et al.* 2007; Krömker and Friedrich 2009) particularly if that quarter had clinical mastitis (Waage *et al.* 2000).

Dry cow therapy with antimicrobials in mature cows is a cornerstone of mastitis control programmes aiming to reduce existing intramammary infections (Dodd *et al.* 1969). Similar treatment administered to heifers pre-partum might reduce the prevalence of intramammary infections at calving and decrease the incidence of clinical mastitis after calving. This would reduce the losses caused by intramammary infections and clinical mastitis in heifers during the first lactation.

Pre-partum treatment of heifers with intramammary products registered for use in mature cows was effective to eliminate existing intramammary infections, reducing the prevalence of clinical mastitis and intramammary infections in early lactation and throughout the first lactation. This has been reported for products intended for use in the dry period (Trinidad *et al.* 1990c; Trinidad *et al.* 1990d; Owens *et al.* 1991, 1994; Owens and Ray 1996; Owens *et al.* 1999; Owens *et al.* 2001; Hovareshti *et al.* 2007; Sampimon *et al.* 2009) or during lactation of mature cows (Oliver *et al.* 1992, 1997; Oliver *et al.* 2003; Oliver *et al.* 2004; Middleton *et al.* 2005; Borm *et al.* 2006; Roy *et al.* 2007).

Evidence from previous studies suggests that the best time for intramammary treatment of heifers is in the last trimester of pregnancy, probably 2 to 16 weeks before expected calving (Trinidad *et al.* 1990c; Oliver *et al.* 1992; Fox *et al.* 1995; Oliver *et al.* 1997; Owens *et al.* 2001; Oliver *et al.* 2003; Oliver *et al.* 2004; Steinman *et al.* 2005; Borm *et al.* 2006). Early treatment probably reduces the damage caused by existing intramammary infections to the secretory tissue in the gland whilst it is rapidly

developing in the late gestation. However, treatment at breeding does not result in higher cure rates than treatment in the second or third trimester of gestation (Trinidad *et al.* 1990c).

The discussion above demonstrates that measures can be taken to start the first lactation with heifers cured from mastitis. This could potentially result in better production and reproductive performance of heifers that were cured from mastitis. However, the effect of intramammary infections in heifers around calving on the future performance of those animals is not yet firmly established. Data of this type are not available for New Zealand dairy herds.

Part Four of this thesis examines the effects of intramammary infections present around calving upon the future performances of those animals, *via* an analysis of the herd test data, mating and clinical mastitis records of heifers on one farm in the Manawatu, New Zealand. Some heifers were treated with a novel product intended for use before calving in heifers. The product has a novel delivery system and works as a temporary internal teat sealant with a delayed release of antimicrobial. The incidence of clinical mastitis, prevalence of subclinical mastitis, milk production and reproductive performance were compared between treated and un-treated (control) heifers (Chapter 10).

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Chapter 10

Treatment before calving of heifers for mastitis improves their reproductive performance, but not their milk production

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10. Treatment before calving of heifers for mastitis improves their reproductive performance, but not their milk production

10.1 Abstract

AIMS: To investigate the effects of treatment before calving for mastitis of heifers on the incidence of clinical mastitis, the prevalence of subclinical mastitis, milk production and reproductive performance over the first lactation.

METHODS: Heifers on a dairy farm in the Manawatu region of New Zealand were treated approximately 4 weeks before planned start of calving with a novel intramammary product containing antimicrobial in a delayed release base acting as a temporary teat sealant (treated group) before calving or not treated (control group). Clinical mastitis after calving was detected by farm personnel recording the identities of affected heifers. Subclinical mastitis after calving was determined as the presence of individual cow somatic cell counts >200,000 cells/mL during routine herd testing. Milk production was estimated from test-day records at routine herd testing on eight occasions throughout lactation. Reproductive parameters (days to first service, number of services per conception and days open) were compared between the treatment groups.

RESULTS: Heifers represented 27.8% of the herd. Of 211 heifers, 21 (10.0%) had one or more episodes of clinical mastitis. They were all from the control group. The prevalence of subclinical mastitis increased over the first lactation from 11.8% to 45.6% in control heifers and 38.1% to 54.4% in treated heifers. Treated heifers had shorter lactations (243.0 ± 6.2 vs. 278.1 ± 2.8 days-in-milk; $P < 0.001$), similar milk volumes (4117.2 ± 141.4 vs. 3912.0 ± 69.6 L; $P = 0.241$) and total milk solids (328.0 ± 10.1 vs. 306.9 ± 4.9 kg; $P = 0.091$) than controls. Treated heifers had fewer days to first insemination (66.3 ± 4.1 vs. 91.6 ± 1.8 , $P < 0.001$) and days open (70.0 ± 5.0 vs. 105.5 ± 2.2 ; $P < 0.001$) and required fewer inseminations per conception (1.2 ± 0.1 vs. 1.6 ± 0.1 ; $P = 0.016$).

CONCLUSIONS: This study demonstrated that treatment of heifers before calving results in less clinical mastitis and improved reproductive performance, but not in increased milk production. Further studies on a larger scale are required to establish the relationship of treatment and milk production in heifers in New Zealand.

KEY WORDS: Mastitis, individual cow somatic cell count, heifers, reproductive performance

ICSCC - Individual Cow Somatic Cell Count

10.2 Introduction

Studies of mastitis in heifers (2-year-old primiparous cattle) date back to the 1930s (Palmer *et al.* 1941; Van Rensburg 1942). Traditionally, heifers have been regarded as mastitis free new introductions into a herd. In the last two to three decades, this belief has been seriously challenged and mastitis in heifers has been identified as a problem. Numerous studies have confirmed that the prevalence of intramammary infections in heifers may be high, up to 80% (Oliver *et al.* 2003; Kalmus *et al.* 2007; Piepers *et al.* 2010). Similar consideration applies to clinical mastitis in heifers early post-calving, in which incidence rates up to 40% have been reported (Edinger *et al.* 1999; Edinger *et al.* 2000; Svensson *et al.* 2006). Studies from New Zealand (Compton *et al.* 2007; Parker *et al.* 2007a; Parker *et al.* 2007b; Parker *et al.* 2008) have shown that the prevalence of intramammary infections in grazing heifers at calving (12 - 49%) and the overall incidence of clinical mastitis (6 - 24%) are lower than in the overseas housed heifers.

Heifers with intramammary infections at calving are at increased risk of udder health problems (De Vlieghe *et al.* 2004), decreased milk yield (King 1967; De Vlieghe *et al.* 2005b; Piepers *et al.* 2010), higher somatic cell count in their first lactation (Trinidad *et al.* 1990b; Hallberg *et al.* 1995; De Vlieghe *et al.* 2005a) and culling (Myllys and Rautala 1995; De Vlieghe *et al.* 2005a; Compton *et al.* 2007). Of the reported studies only Compton *et al.* (2007) was carried out in New Zealand. Consequently, affected heifers have decreased value to the herd and potentially shortened longevity (Druet *et al.* 2003; Heringstad *et al.* 2003; Bar *et al.* 2007). No data on the effects of mastitis in heifers on milk production are available for pasture-based systems such as those in New Zealand. Milk losses would be expected to be similar to those observed elsewhere.

Several studies have shown that the reproductive performance of mature cows with clinical or subclinical mastitis is impaired (Barker *et al.* 1998; Hockett *et al.* 2000; Schrick *et al.* 2001; Chebel *et al.* 2004). The association of udder health at calving and reproductive performance in heifers has not been investigated in detail (Nava-Trujillo *et al.* 2010). It is postulated that the association between subclinical mastitis and

reproductive parameters would be comparable to that in mature cows.

This longitudinal prospective clinical study investigated the incidence of clinical mastitis, prevalence of subclinical mastitis, milk production and reproductive performance in heifers over the first lactation that were treated with a novel intramammary product before calving or were non-treated on a farm in the Manawatu region of New Zealand.

10.3 Materials and methods

The design of the animal phase of this study was approved by Kaiwhina Animal Ethics Committee (KAEC 006/09).

The collaborating farm was selected on the basis of a regular herd testing, a history of keeping accurate mastitis treatment records and the share-milker's consent. Usual farming practices and feeding were followed during the study period. The herd was milked twice daily. Herd records were kept electronically on the farm.

Herd tests were carried out on 8 occasions through the lactation on following dates in August, September, October, November, December, January, March and May. The herd test included milk volume, percent and mass of milk fat and protein, somatic cell count. The records for each variable represent the test-day values. Herd test data were available for 211 heifers (35 treated and 176 control heifers). Herd test data were used to estimate the prevalence of subclinical mastitis. Ten heifers with herd test data had no records of mating. Hence, mating records were available for 201 heifers (33/35 treated and 168/176 control heifers). Mating records were used in estimating the reproductive parameters.

Clinical mastitis was managed by farm personnel. Cows that had quarters diagnosed with mastitis by farm personnel (based on a standard operating procedure looking for swelling, redness, heat and pain on touch and changes in secretion – clots, colour or consistency) were recorded, including the heifer's identification, affected quarter treatment/s and outcome.

10.3.1 Procedures

A cohort of 35 heifers was treated with one injector per quarter of a novel product intended for use in heifers before calving. The product has a novel delivery system and

works as a temporary internal teat sealant with a delayed release of antimicrobial. Treatment was carried out approximately 4 weeks (range 3 - 12 weeks) before planned start of calving. Control heifers remained un-treated. The number of treated heifers allowed for estimation of the effects of treatment before calving on the incidence of clinical mastitis after calving (statistical power of 89.4%).

Diagnosis of clinical mastitis in affected quarter/s was undertaken by farm personnel using the procedures that were currently practiced on the farm.

10.3.2 Statistical Analysis

Statistical analyses were carried out using SAS (Statistical Analysis System; *SAS Institute Inc.*, Cary, NC, USA) version 9.2 and Excel (Microsoft Office; *Microsoft Corporation*, USA) version 2010.

Days-in-milk included the period from calving to drying off.

The total rate of clinical mastitis was calculated as the cumulative incidence (new and recurrent cases) per 100 heifers. The effect of treatment on the incidence of clinical mastitis was estimated using the paired difference t-test.

Subclinical mastitis (*i.e.* intramammary infection without observed clinical signs) per treatment group was estimated from the test-day individual cow somatic cell count (ICSCC) available from the herd testing data. A heifer had a high ICSCC when test-day ICSCC $\geq 200,000$ or low when test-day ICSCC $< 200,000$ somatic cells/mL. In New Zealand, the National Mastitis Advisory Committee has advised lower cut-off values of 150,000 for mature cows and 120,000 cells/mL for heifers. A new intramammary infection was defined as presence of low ICSCC at one herd test followed by high ICSCC at the next herd test. A cure from intramammary infection was defined as the presence of high ICSCC at one herd test followed by low ICSCC at the next herd test. If there was no change in the status of the ICSCC level then the heifer remained in the same category (*i.e.* contributed to the denominator only).

The lactation curves and somatic cell curve per group were modelled using the Ali-Schaeffer curves (Ali and Schaeffer 1987).

Days to first service included the period from calving to first insemination. The number of insemination attempts to result in a confirmed conception was regarded as number of

services per conception. Days open included the period from calving to the insemination that resulted in a confirmed conception. The effect of treatment on reproductive parameters (days to first service, number of services per conception and days open) was estimated using the paired difference t-test. The effect of high ICSCC at the first, second or third herd test, which occurred before the median mating date, on the reproductive performance parameters was also estimated using the paired difference t-test.

The level of significance was set at $P < 0.05$.

10.4 Results

Heifers comprised 27.8% (211/785) of cows in the herd. The average age in the herd was 4.6 years (range 2 – 14).

10.4.1 Incidence of clinical mastitis

The cumulative incidence was 31/211 episodes (14.1%) of clinical mastitis in 21/211 heifers (10.0%). All episodes were in single quarters in heifers from the control group. The rate of clinical mastitis in the control heifers ($18.4 \pm 2.7\%$) was significantly higher than in treated heifers ($0 \pm 8.1\%$; $P=0.031$). Recurring cases of clinical mastitis were present in 8/21 (38.1%) heifers.

10.4.2 Prevalence of subclinical mastitis

Records were available for 1364 heifer-data-points of 211 heifers at 8 herd tests. The prevalence of subclinical mastitis was highest in the early season, reached nadir at peak lactation and thereafter, increased continuously through the remainder of the lactation (Table 10.1). The increase in the ICSCC (geometric means) was greater for the treated heifers (Figure 10.1). No difference was found in the rate of new intramammary infections between treated ($71.4 \pm 12.8\%$) and control ($70.5 \pm 5.7\%$) heifers over the course of the first lactation ($P=0.95$). In contrast, there was a difference in the cure rates of intramammary infections between the treated ($54.3 \pm 10.7\%$) and control ($35.8 \pm 4.8\%$) heifers ($P=0.001$). No differences between the treated (76.7%) and controls (75.6%) were observed in the percentage of heifers with low ICSCC at the first herd test after calving that remained low until the end of the season ($P=0.146$). However, the number of heifers with high ICSCC at the first herd test in treated heifers was significantly higher ($P < 0.01$) than in untreated heifers.

Table 10.1. Changes in the percentage of heifers with high individual somatic cell counts (High ICSCC; SCC \geq 200,000/mL) from low to high (New infections) and high to low (Cured infections) approximating rates of new infections and cures from subclinical mastitis and the percentage of high ICSCC on test-day in treated and control heifers during their first lactation

Herd test	New infections			Cured infections			High ICSCC		
	Control	Treated	Statistical difference ¹	Control	Treated	Statistical difference	Control	Treated	Statistical difference
1	-	-	-	-	-	-	11.8	38.1	0.001
2	3.3	9.5	0.173	2.6	23.8	<0.001	12.5	24.2	0.08
3	6.0	6.3	0.949	6.0	15.6	0.057	13.2	14.7	0.817
4	9.8	15.2	0.367	5.2	0.0	0.182	17.7	30.3	0.096
5	5.2	3.0	0.601	6.9	12.1	0.305	15.5	21.2	0.421
6	11	9.1	0.749	4.0	6.1	0.606	22.5	24.2	0.832
7	18.5	24.2	0.447	6.4	3.0	0.457	34.7	45.5	0.241
8	21.6	23.5	0.858	7.4	11.8	0.533	39.8	25.7	0.118
Total	45.4	54.6	0.589	34.7	65.3	0.311	45.6	54.4	0.366

¹Statistical difference (P-values) indicate the difference between the corresponding group values within row.

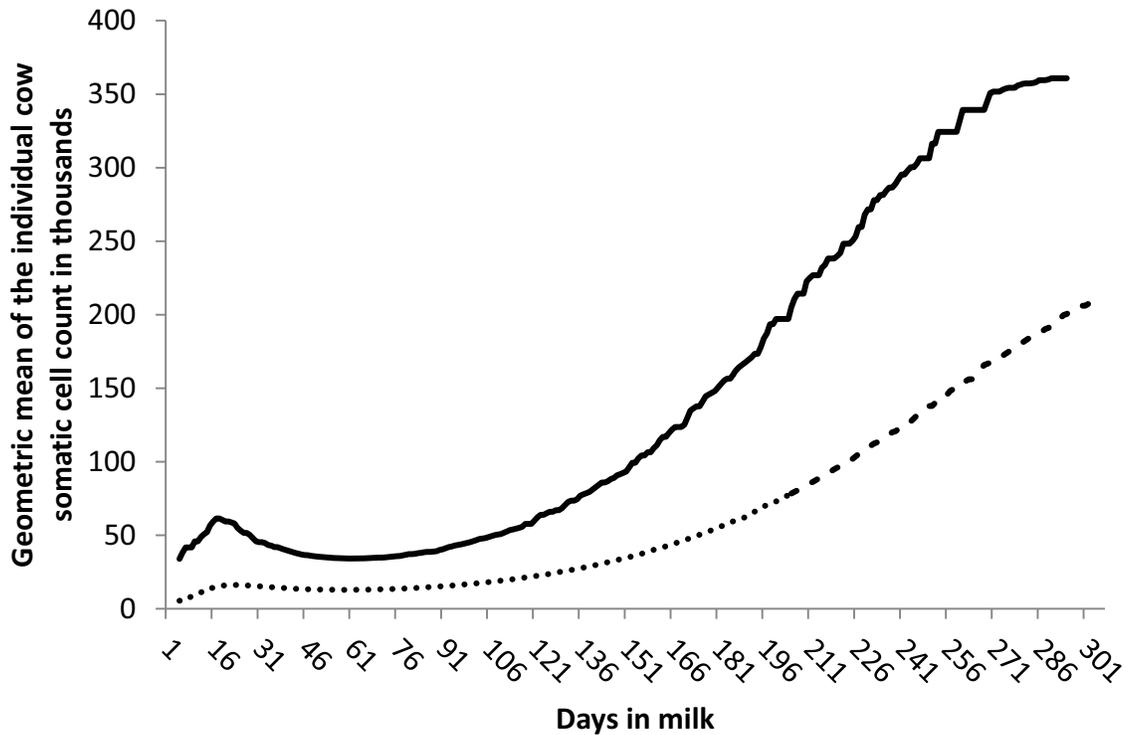


Figure 10.1. Moving average (10-daily) of the predicted geometric mean of individual cow somatic cell count in treated (continuous line) and control heifers (dotted line) in their first lactation

‘Day 1’ = Day of calving, ‘Day 2’ = Days 1-2 smoothed over Day 1, ‘Day 3’ = Days 1-3 smoothed over Days 1-2, ‘Day 4’ = Days 1-4 smoothed over Days 1-3, ‘Day 5’ = Days 1-5 smoothed over Day 1-4, ‘Day 6’ = Days 1-6 smoothed over Days 1-5, , ‘Day 7’ = Days 1-7 smoothed over Days 1-6, , ‘Day 8’ = Days 1-8 smoothed over Days 1-7, , ‘Day 9’ = Days 1-9 smoothed over Days 1-8, , ‘Day 10’ = Days 1-10 smoothed over Days 1-9, , ‘Day 11’ = Days 2-11 smoothed over Days 1-10, etc.

The prevalence of either subclinical mastitis or new infections in treated heifers over the first lactation was variable and did not follow a trend (Figure 10.2). New intramammary infections in treated heifers were acquired in 16 (45.7%), 3 (8.6%) and 1 (2.9%) heifers 1, 2 or 3 times, respectively. Cure from intramammary infection occurred in 7 (20.0%) and 6 (17.1%) heifers cured 1 or 2 times, respectively.

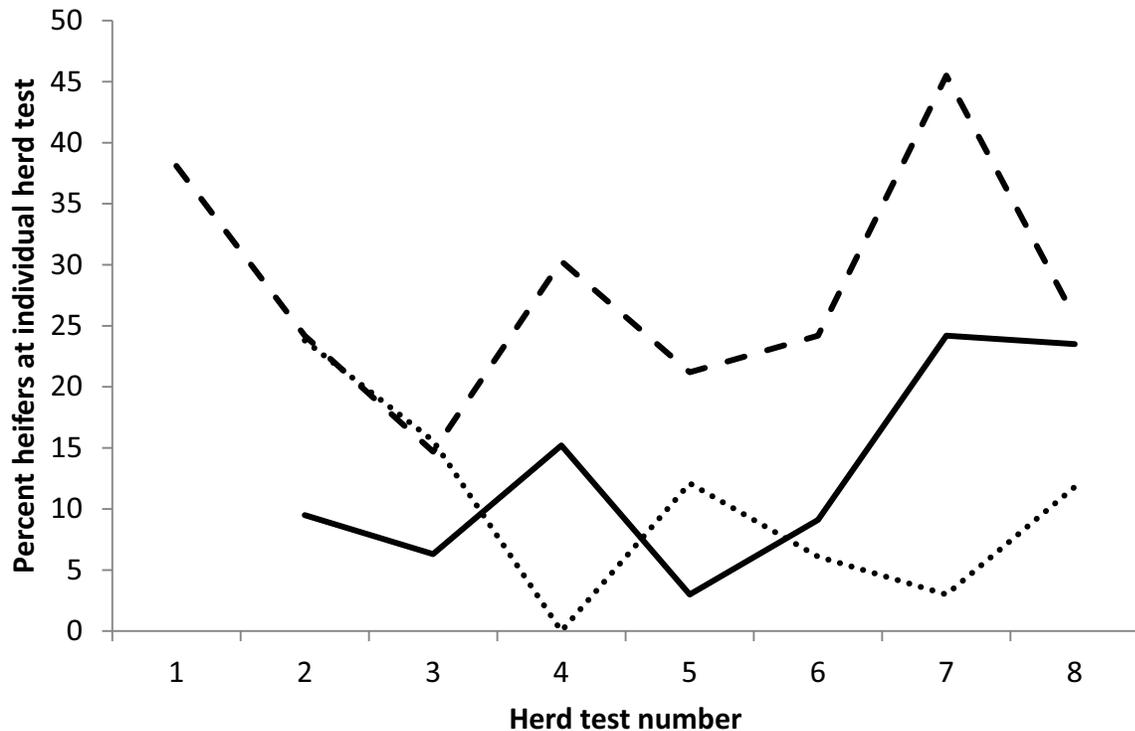


Figure 10.2. Percentage of treated heifers with high individual cow test-day somatic cell count (ICSCC $\geq 200,000/\text{mL}$; dashed line) percentage of new infections (change of ICSCC from low to high; solid line) and percentage of cured cases (change of ICSCC from high to low; dotted line) through their first lactation

The prevalence of subclinical mastitis and of new intramammary infections in control heifers increased steadily in parallel with each other over the course of the first lactation. Conversely, the cure rates for intramammary infections in control heifers were consistent throughout the first lactation (Figure 10.3). New intramammary infections were acquired in 72 (40.9%), 20 (11.4%) and 4 (2.3%) heifers 1, 2 or 3 times, respectively. Cure from intramammary infections occurred in 44 (25.0%), 8 (4.6%) and 1 (0.6%) heifers 1, 2 or 3 times, respectively.

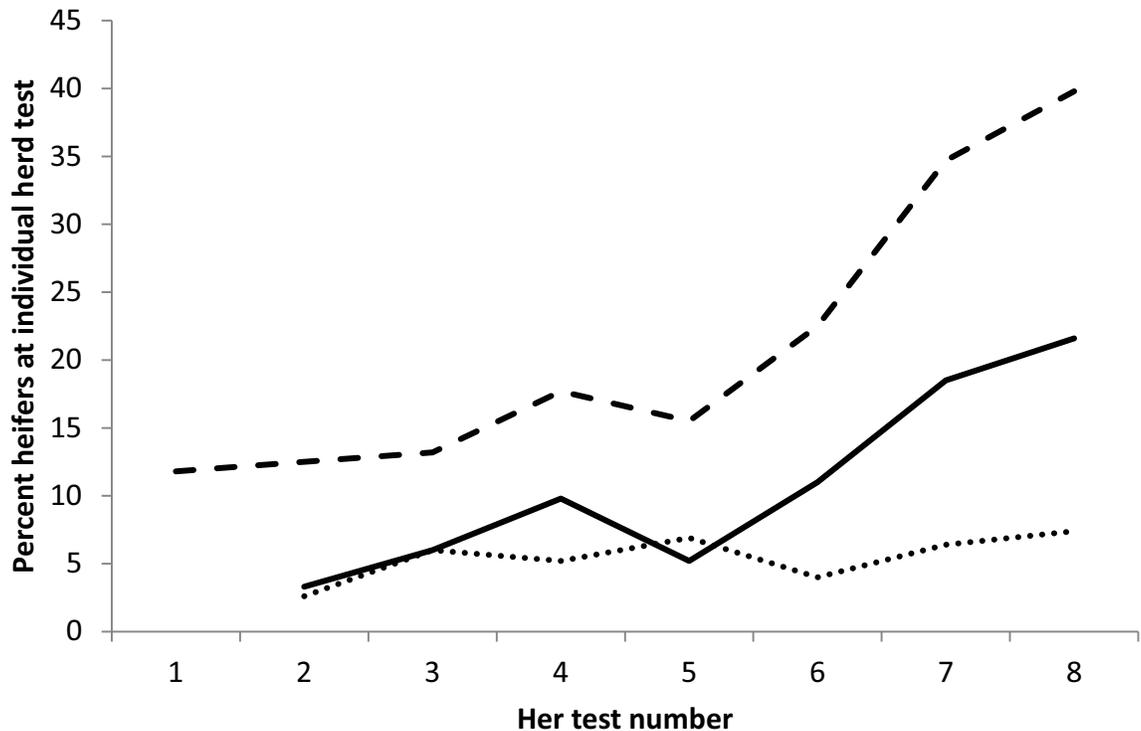


Figure 10.3. Percentage of control heifers with high individual cow test-day somatic cell count (ICSCC \geq 200,000/mL; dashed line) percentage of new infections (change of ICSCC from low to high; solid line) and percentage of cured cases (change of ICSCC from high to low; dotted line) through their first lactation

10.4.3 Days-in-milk

The median calving date for treated heifers was 18 August 2008 and for control heifers was 29 July 2008. The spread of calving was 10 weeks for treated heifers and just over 21 weeks for control heifers. The total days-in-milk of the treated heifers (243.0 ± 6.2) was significantly shorter than of control heifers (278.1 ± 2.8 ; $P < 0.001$).

10.4.4 Milk production

The milk volume and milk solids were adjusted for the effect of the total days-in-milk. The total milk volume of treated (4117.2 ± 141.4 L) and control (3912.0 ± 69.6 L) heifers were similar ($P = 0.241$). Also, there was no difference in the total milk solids of treated (328.0 ± 10.1 kg) and control (306.9 ± 4.9 kg) heifers ($P = 0.091$). Lactational curves for both groups of heifers were similar, despite the greater variability in the lactation curve of control heifers (Figure 10.4).

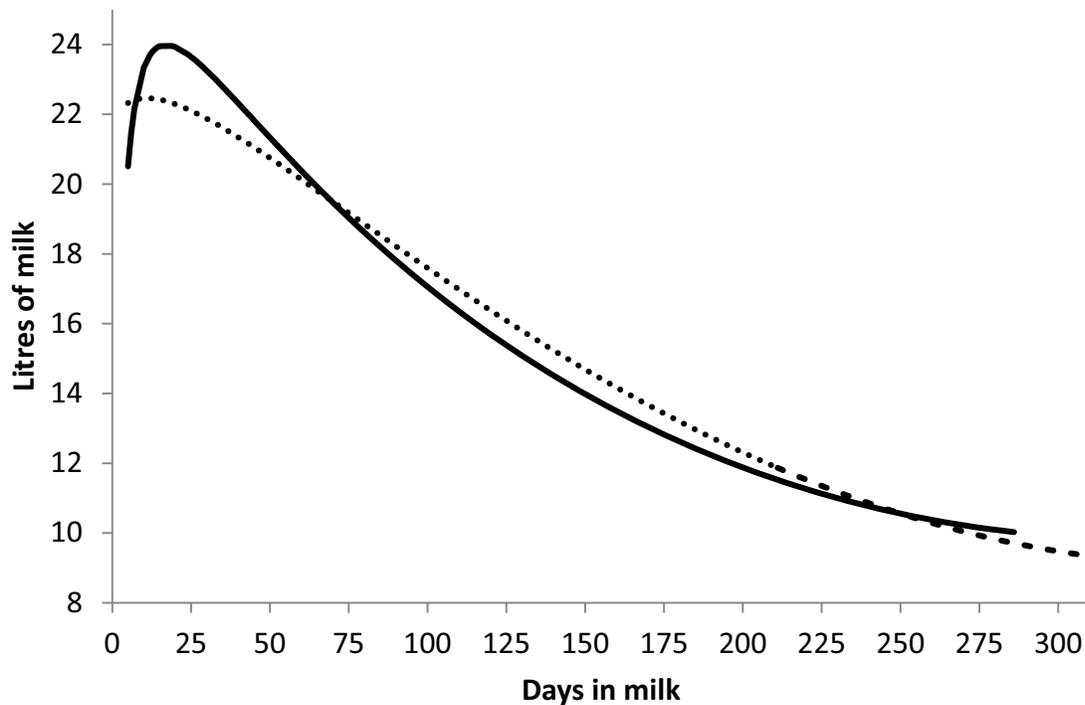


Figure 10.4. Predicted milk volume using the method of Ali and Schaeffer (1987) in treated (continuous line) and control heifers (dotted line) during their first lactation

10.4.5 Reproductive performance

A summary of the reproductive parameters for heifers is presented in Table 10.2. The reproductive performance of heifers was poorer than for the mature cows. Measured reproductive performance of treated heifers was significantly better than the controls.

No relationship between the high ICSCC and reproductive performance was observed ($P>0.05$).

Table 10.2. Means \pm SE and differences of three reproductive parameters in treated and control heifers

Reproductive parameter	Treated	Control	Difference
Days to first service	66.3 \pm 4.1	91.6 \pm 1.8	<0.001
Number of inseminations	1.2 \pm 0.1	1.6 \pm 0.1	0.016
Days open	70.0 \pm 5.0	105.5 \pm 2.2	<0.001

10.5 Discussion

This study of the effects of treatment of heifers before calving on the incidence of clinical mastitis, prevalence of subclinical mastitis, milk production and reproductive performance in the first lactation is first of its type in New Zealand. The study demonstrated a lower incidence of clinical mastitis, higher prevalence of subclinical mastitis, similar milk production and better reproductive performance in treated heifers.

The dataset, which was based on farm records, has some limitations. Firstly, the occurrence of clinical mastitis obtained from farm records is influenced by the farmer's ability to detect and record cases. The detection rate is affected by the diagnostic criteria of each member of the farm staff. The accuracy of recording is affected by the diligence with which records are kept and entered into the data-base. This study was carried out on a single farm with known excellent record keeping. Hence, it is unlikely that diagnostic criteria or record keeping changed over the duration of the study and no diagnostic and recording bias would be introduced. Secondly, no information on the types of mastitis-causing organisms was available. However, the study did not address pathogen-specific effects in the analysis. Therefore, this is a minor limitation applicable for researchers dealing with the prevalences of various mastitis-causing organisms for mastitis in heifers. The study was carried out on a single farm, but the statistical power was sufficient to show the differences between treated and control heifers (calculations of statistical power not shown). Treatment used in this study was novel and relied on delayed release of antimicrobial. It is unknown if the results obtained in this study are comparable to those where treatment was carried out with conventional lactating or dry cow products.

The importance of heifers for farm sustainability is emphasised by the fact that heifers are the largest parity group on many farms in New Zealand. The mean percentage of heifers in the Waikato and Taranaki regions of New Zealand was 17.8% (Parker *et al.* 2007b) and 22.7% in 14 herds from Northland in 2005/06 (Petrovski *et al.*, unpublished). In the present study, heifers represented 27.8% of cows in the herd. Furthermore, heifers usually have the highest genetic merit of any age group on the farm. Therefore, any disorders with the potential to affect lifetime productivity of heifers must be taken seriously.

The rate of clinical mastitis of 14.1% was within the range of previous reports from New Zealand of 6.7 - 23.4% (Compton *et al.* 2007; Parker *et al.* 2007a; Parker *et al.* 2007b).

However, any comparison with these studies should be made cautiously as these reported the incidence of clinical mastitis in the peripartum period only. It would be better if data was comparable. However, no other references were found in the literature that contained a similar method of calculation to that of the present study. In 14 farms and records of 3765 cows in Northland the cumulative incidence of clinical mastitis in heifers for the season 2005/06 was 16.9% (Petrovski *et al.*, unpublished). It is likely that the cumulative incidence of clinical mastitis in heifers in this study would have been higher if the treatment cohort of heifers had not been treated before calving. This is supported by the higher rate of clinical mastitis in control heifers of 18.4%. Therefore, the incidence of clinical mastitis in heifers in New Zealand is likely higher compared to herd level of approximately 15% (McDougall 1999, 2007; Petrovski *et al.* 2009). Thus, treatment of heifers before calving prevented occurrence of clinical mastitis in their first lactation. This is in agreement with reports elsewhere (Oliver *et al.* 2003; Borm *et al.* 2006; Sampimon *et al.* 2009).

The negative correlation between the level of somatic cells and milk yields in cattle is well established for mature cows (Miller *et al.* 1983; Jones *et al.* 1984; Salsberg *et al.* 1984) and heifers (Miller *et al.* 1993). Unlike the situation in mature cows, the level of somatic cell count in milk of uninfected heifers is not influenced by days-in-milk (Laevens *et al.* 1997; Schepers *et al.* 1997). Therefore, uninfected quarters should remain with a low ICSCC throughout the first lactation. In this study approximately half of the heifers remained with a low ICSCC over the course of the first lactation (Table 10.1). Numerous authors (Coffey *et al.* 1986; De Vliegher *et al.* 2004; Whist *et al.* 2007; Paradis *et al.* 2010) have shown that heifers with low somatic cell count early in lactation had significantly lower somatic cell count throughout their first lactation. This trend was also detected in this study.

Despite the apparent better cure rates in the treated heifers, the overall rates of intramammary infections in late lactation for both groups were the same. The rate of new intramammary infections was higher in the late lactation, being more than 15% in both groups (Figures 10.2 and 10.3). This may indicate a high infection pressure in the herd and the effect of various levels of intramammary infection on herd level on the rate of new intramammary infections in heifers warrants a further investigation. However, the rate of high ICSCC in treated heifers in early lactation was higher than in controls. This finding is in contrast to previous reports that demonstrated significantly lower rate of

intramammary infections in treated heifers in early lactation (Trinidad *et al.* 1990a; Owens and Ray 1996; Oliver *et al.* 2003). The reason for the high rate of intramammary infections in treated heifers at the first herd test for the season is unclear. It is possible that treatment administered to the treated heifers caused a local irritation and they started the season with higher ICSCC. Heifers calving with higher ICSCC remained higher for the remaining of the lactation (De Vlieghe *et al.* 2005a; Whist *et al.* 2007; Paradis *et al.* 2010). This study confirmed this notion because higher ICSCCs were present throughout the first lactation in the treated heifers which started the lactation with higher ICSCC. It was not possible to compare the finding of higher ICSCC at calving in treated heifers to previous reports as the type of treatment differed.

Milk volume and milk solids production in the first season were similar for both groups of heifers. The presence of transient intramammary infections in heifers which are cleared shortly after calving is associated with negligible production losses (De Vlieghe *et al.* 2004; De Vlieghe *et al.* 2005a; Piepers *et al.* 2010). Furthermore, New Zealand data indicate that there are no long-term significant losses of milk production, most likely because most intramammary infections were caused by transient environmental infections with streptococci (Compton *et al.* 2007). It was, therefore concluded that the short-term increase of the ICSCC at calving in this study had no negative effect on milk production. However, if there was no increase in the ICSCC at calving, it would be expected the milk volume and milk solids production in treated heifers to be higher than controls, as found elsewhere (Oliver *et al.* 2003).

Clinical mastitis before or around the time of insemination results in decreased conception rate, increased days to first service and increased number of services per conception (Schrick *et al.* 2001; Santos *et al.* 2004; Nava-Trujillo *et al.* 2010). If subclinical mastitis before or around the time of insemination has any effect is more controversial. Negative effects on reproductive performance were reported from some studies (Schrick *et al.* 2001; Hockett *et al.* 2005; König *et al.* 2006) and not from others (Miller *et al.* 2001; Klaas *et al.* 2004). The variability in the results is likely because the effects of intramammary infections before or around insemination differ between cows as it has been demonstrated that about 30% of cows react with a delayed ovulation (Lavon *et al.* 2010). The variation in the ovulation between cows and heifers may result of the low genetic correlation of mastitis to reduced fertility of 0.21 - 0.41 (Ødegård *et al.* 2003; Andersen-Ranberg *et al.* 2005; Heringstad *et al.* 2006). In the present study, treated

heifers had a significantly better reproductive performance than did the control heifers. The improved reproductive performance in the first lactation would result in better milk production and a tighter calving pattern the following year, which in turn should result in better reproductive performance in the second lactation. Thus, treatment of heifers before calving may help their life-long productivity. This unexpected observation is an area that requires further research.

10.6 Conclusions

This study demonstrated that treatment of heifers before calving results in improved reproductive performance, but not in increased milk production. Treatment of heifers before calving resulted in no clinical mastitis in this group throughout the first lactation. The treatment caused a temporary increase in the ICSCC and a rapid development of subclinical mastitis in heifers that had a high ICSCC early post-calving. Further studies on a larger scale are required to establish the relationship of treatment and milk production in heifers in New Zealand.

10.7 Acknowledgments

Financial support from *Bomac Ltd*, Auckland, New Zealand is greatly appreciated. Thanks to the staff at *Penshurst Farms* and share-milkers John and Wendy Allen and staff at *Estendard Ltd* for their involvement in the animal phase of the study. KR Petrovski's position at Massey University is supported by *Bomac a company of Bayer Ltd*.

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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Kiro R Petrovski

Name/Title of Principal Supervisor: Prof Norman B Williamson

Name of Published Paper: Treatment before calving of heifers for mastitis improves their reproductive performance, but not their milk production

In which Chapter is the Published Work: Chapter 10

What percentage of the Published Work was contributed by the candidate: 80%



Candidate's Signature

11 July 2011

Date



Principal Supervisor's signature

14/7/2011

Date

Part 4

Antimicrobial teat sealant for use at drying
off

Chapter 11

Introduction to Part Four: antimicrobial teat sealant for use at
drying off

11. Introduction to Part Four: antimicrobial teat sealant for use at drying off

In Part Four of the thesis an experimental challenge study design is used to test the efficacy of an internal teat sealant containing 0.5% chlorhexidine in the reduction of incidence of intramammary infections in the dry period. While the treatment of intramammary infections during lactation is important for shortening the shedding phase (Dodd *et al.* 1969), preventing the incidence of intramammary infections will have the most significant effect on lowering the prevalence of mastitis in most dairy herds. This is particularly important for New Zealand's seasonal dairying where all cows on a farm are dry at once, so the additional complication of spread during milking (of contagious mastitis-causing organisms) is taken out of the equation. In other words, if management of the dry period can result in fewer infected cows at calving, provided other mastitis management measures are in place, there should be a significant impact upon the prevalence of mastitis during the subsequent lactation.

This part starts with a critical overview of publications (Chapter 11) addressing internal teat sealants. Chapter 12 describes a preliminary investigation of the efficacy of two formulations of a novel, chlorhexidine-containing, internal teat sealant against an experimental challenge by *Streptococcus uberis* in dry cows. Chapter 13 describes a similar, large-scale study with a preferred formulation of the chlorhexidine-containing teat sealant.

The differences between these two studies are that in the preliminary investigation two formulations of the novel, chlorhexidine-containing, teat sealant were evaluated and compared to positive and negative control cows. The experimental design was based on treating all four quarters in a cow with the same product, whilst negative control cows remained untreated. In the second study, treated cows were used to provide within-cow controls, as two contra-lateral quarters were treated with the chlorhexidine-containing teat sealant, and remaining two were treated with the conventional teat sealant (Figure 11.1). Additional cows served as untreated controls.

TS	ATS	ATS	TS
ATS	TS	TS	ATS

Figure 11.1. Treatment design for the second challenge study (Chapter 13). ATS ~ novel chlorhexidine-containing teat sealant (*Bomac Ltd*, Auckland, New Zealand); TS-Teatseal™ (*Pfizer Animal Health*, Auckland)

The importance of the dry period for bovine mastitis control programmes has been elaborated in numerous reviews (Neave *et al.* 1950; Eberhart 1986; Dingwell *et al.* 2003a; Bradley and Green 2004) and a short description of the important factors during the dry period that affect susceptibility to intramammary infections was addressed in the general introduction to this thesis (Chapter 1).

The goal of mastitis control during the dry period is to have fewer infected quarters at the next calving. Achieving this involves eliminating existing subclinical intramammary infections that are present at the end of lactation, and preventing the occurrence of new infections during the dry period (Neave *et al.* 1966; Philpot 1979; Funk *et al.* 1982; Eberhart 1986; Browning *et al.* 1990; Browning *et al.* 1994; Hassan *et al.* 1999; Berry and Hillerton 2002a, 2002b; Dingwell *et al.* 2002; Dingwell *et al.* 2003b). Prevention of new intramammary infections was suggested to be of greater long-term benefit to the dairy industry (Eberhart 1986) than the treatment during lactation.

At present, the most effective means of achieving the lowest prevalence of infected quarters at calving and a key component of any dry-cow management strategy is the use of antimicrobial dry cow therapy (DCT) and/or the use of teat sealants after the last milking (Philpot 1979; Eberhart 1986; Bradley and Green 2004). However, these methods are not totally efficacious and a few new intramammary infections may still occur during the dry period.

New intramammary infections may occur throughout the dry period when mastitis-causing organisms are not susceptible to the antimicrobial contained in the DCT. This is common with coliform organisms (Eberhart 1986). Another shortcoming of existing antimicrobial DCT is the diminished or absent protection against new intramammary infections in the late dry period when concentrations of the antimicrobial compound in the udder are declining (Smith *et al.* 1985; Eberhart 1986; Bradley and Green 2001).

Teatseal™ (*Pfizer Animal Health*, Auckland, New Zealand; the same product overseas is known as Orbaseal®) is a viscous non-antimicrobial formulation that forms an internal plug when infused hygienically into the teat canal sinus at the time of dry-off. Such a plug provides a physical barrier to invasion by mastitis-causing organisms throughout the dry period (Meaney 1977; Woolford *et al.* 1998; Ryan *et al.* 1999; Twomey *et al.* 2000; Huxley *et al.* 2002; Godden *et al.* 2003; Crispie *et al.* 2004a; Hillerton and Berry 2004; Crispie *et al.* 2005). Teat sealants are given as a single administration at drying off and have to be removed by manual stripping at calving. There is no evidence of any chemical binding to the wall of the teat cistern. All data indicate that it is purely a physical barrier. For example, Williamson (2001) stated that conventional internal teat sealants fill the fissures and folds within the teat canal and teat sinus.

Since their initial development in the 1970s, internal teat sealants, containing bismuth sub-nitrate, have been evaluated in Canada, Ireland, New Zealand, the United Kingdom and the USA (Table 9.1). These studies have demonstrated that application to uninfected mammary gland is as effective as using a long-acting antimicrobial, in terms of achieving control of the rate of new intramammary infections during the dry period. They are beneficial in mastitis control and produce an economic return in comparison to non-treatment (Meaney 1977; Woolford *et al.* 1998; Berry and Hillerton 2002b; Godden *et al.* 2003; Crispie *et al.* 2004a, 2004b; Berry *et al.* 2004; Hillerton and Berry 2004).

Studies using Teatseal™ have demonstrated a significant reduction in new intramammary infections during the dry period in treated quarters compared to untreated quarters (Woolford *et al.* 1998), the same or better efficacy when used in combination with antimicrobial DCT compared to DCT alone (Woolford *et al.* 1998; Berry and Hillerton 2002b; Huxley *et al.* 2002; Hillerton and Berry 2004; Cook *et al.* 2005; Berry and Hillerton 2007; Newton *et al.* 2008; Bradley *et al.* 2010), and the same or better efficacy when used combination with bacteriocins compared to teat sealant alone (Ryan *et al.* 1998; Crispie *et al.* 2004b; Crispie *et al.* 2005). Furthermore, a significant reduction in clinical mastitis in the first 100 days of the next lactation has been observed in most studies of quarters sealed at drying-off (Woolford *et al.* 1998; Williamson 2001; Berry and Hillerton 2002b; Huxley *et al.* 2002). This reduction is postulated to be due to a fewer of subclinical infections acquired in the dry period and persisting into the following lactation (Williamson 2001). One of the main advantages in combining the seal with a broad-spectrum bacteriocin is that, in addition to the barrier protection of the

seal, the seal also localises the microbial inhibitor in the teat sinus (Ryan *et al.* 1999; Crispie *et al.* 2004b).

Teatseal™ is not ideal in the prevention of new IMIs during the dry period since a failure of 2 - 34.6% has been reported in several studies (Table 11.1). Moreover, since most of these studies were conducted in controlled situations, it may be reasonable to envisage that failure rates under field conditions may be higher. The most likely reasons for new intramammary infections occurring in the dry period are related to the danger of contamination of introduction of new intramammary infections at administration because of the lack of hygiene and absence of constituents with antimicrobial properties. An aseptic technique is a clear recommendation for the infusion of all intramammary products, but it is known that farmer's practices vary widely, possibly on their assumption that it is less important when infusing antimicrobial formulations (Woolford *et al.* 1998). Farmers may have more confidence in the product if some antimicrobial is incorporated into the treatment. However, this should not replace good hygiene at administration times. The second period new intramammary infections may occur is in the late dry period and colostrogenesis when the teats engorge and the plug formed by the Teatseal™ is loosened from the teat wall leaving a gap for the invading mastitis-causing organisms to enter the teat and mammary gland sinus.

Review of the characteristics of an ideal internal teat sealant was carried out considering for directions of improvement of the conventional teat sealant (review not shown; it contains confidential information). Combining the internal teat sealant with compound/s possessing antimicrobial activity was identified as a direction for improvement. This compound should have local activity against invading mastitis-causing organisms in the late dry period. The antimicrobial spectrum of activity of the compound to be incorporated in the novel sealant required efficacy against most mastitis-causing organisms. Chlorhexidine, possesses activity against most Gram-positive bacteria and also some other infectious organisms (Heit and Riviere 2009) and was assumed to be an ideal candidate. Therefore, an improved, novel product was formulated containing 0.5% chlorhexidine.

Table 1.1.1. Summary of studies on the efficacy of teat sealants alone and their use in combination with other products

Treatment	New IMI during the dry period			Reference	Design
	un- treated	TS ¹ +AM ²	TS		
Cows	32		32		Free of IMI ³
Percent infected	63 ³		25 ³	Patent	SD ⁴ -challenged
Cows	528	528	528	Patent	Free of IMI
Percent infected	16.1	2.5	1.9	2.7	
Cows			928	Huxley <i>et al.</i>	
Percent infected			34.6 ⁷	(2002)	Low SCC ⁵
Cows	528	505	505	Woolford <i>et al.</i>	Low SCC cows
Percent infected	16.1	2	2.5	(1998)	Free of IMI
Quarters	31		31	Meany - unpub	Free of IMI ⁷
Percent infected	65 ³		26 ⁶		SD ⁶ -challenged
Quarters	799		784	Berry & Hillerton	No IMI at dry-off
Percent infected	11.64		3.4	(2002a)	
Quarters d		821	812	Godden <i>et al.</i>	No clinical infection
Percent infected		20.2	25.4	(2003)	IMI rate (by culture) 31% 33% for the 2 groups respectively
Quarters*		1009	971	Cook <i>et al.</i>	Blanket DCT
Percent infected		8.0	16.5	(2005)	
Cows		1010	1003	Runciman <i>et al.</i>	CM ⁸ in whole season
Percent infected		16.7	29.2	(2011)	

¹ TS= teatseal; ² AM= Intramammary antibiotic infusion; ³IMI= Intramammary infection; ⁴SD= *Streptococcus dysgalactiae*; ⁵SCC= Somatic Cell Count; ⁶clinical infection occurred within 10 days; ⁷ calculated from numbers of culture isolates; ⁸Clinical mastitis.

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Chapter 12

A preliminary evaluation of the efficacy of two novel internal teat sealant formulations against bacterial challenge in the early dry period

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12. A preliminary evaluation of the efficacy of two novel internal teat sealant formulations against bacterial challenge in the early dry period

12.1 Abstract

AIMS: The efficacy of two test formulations being evaluated for use as an internal teat sealant against experimental microbial challenge with *Streptococcus uberis* was compared to that from a commercial teat sealant and with negative controls. Treatment was administered at drying off to 14 cows per group while 6 cows were negative controls. Additionally, any irritation caused by the test products was evaluated.

METHODS: Treated cows received intramammary infusion of product, all cows were challenged by dipping the teats in a bacterial broth two and four days after treatment then carefully inspected and udders palpated for 34 days after drying-off. All cases of clinical mastitis were sampled and treated. Milk samples were collected aseptically after calving for culture and determination of somatic cell count. Irritation was observed by the daily palpations and somatic cell count after calving.

RESULTS: Significant protection from infection occurred in all treated quarters and no chronic irritation was caused by the products. The incidence of clinical mastitis in untreated quarters was 46% and under 2% in treated quarters. Most mastitis cases were caused by the challenge strain of *Strep. uberis*. Early in the subsequent lactation the somatic cell counts were lower for groups treated with the two test formulations of the novel internal teat sealant.

CONCLUSION: The novel internal teat sealant demonstrated efficacy at least equivalent to a commercial product in protecting against intramammary infection in the early dry period.

CLINICAL RELEVANCE: Internal teat sealants are important tools for mastitis management on farms. Inclusion of an antimicrobial compound in the novel teat sealant should decrease the risk of intramammary infections introduced at treatment becoming established.

KEY WORDS: experimental challenge, internal teat sealant, mastitis, *Streptococcus uberis*

CAMP - Christie–Atkins–Munch-Petersen test, PFGE – Pulse Field Gel Electrophoresis, SCC – Somatic Cell Count, SCS - Somatic Cell Score

12.2 Introduction

The prevalence of intramammary infections on dairy farms that calve seasonally reflects the incidence rate and the duration of each infection, divided by the number of lactating cows present on the farm for the season. The incidence rate of intramammary infections is highest during the dry period in the absence of antimicrobial dry cow therapy (Neave *et al.* 1950; Cousins *et al.* 1980; Funk *et al.* 1982). Therefore, reducing the incidence of intramammary infections in the dry period should help decrease mastitis prevalence. Non-antibiotic internal teat sealants are widely used within the dairy industry to reduce the incidence of intramammary infection during the dry period and consequently decrease the incidence and prevalence of clinical mastitis after calving (Woolford *et al.* 1998; Bradley and Green 2004; Crispie *et al.* 2004). However, the available teat sealant did not provide complete protection (Woolford *et al.* 1998). Anecdotally, that product is perceived by farmers as difficult to handle due to the risk of introducing new intramammary infections during administration. New products were being developed containing chlorhexidine to prevent or treat bacterial infections in the teat canal and the lower teat sinus at and immediately after the time of treatment at drying off. Two novel formulations were tested in this study cows at drying off.

The efficacy of internal teat sealants is best evaluated by estimating their protective role against natural or experimental challenge with mastitis-causing organisms. This study aimed to determine the efficacy against experimental microbial challenge of two test formulations of novel internal teat sealants containing chlorhexidine when administered to dairy cattle at drying-off and to evaluate any irritation caused compared with negative controls.

12.3 Materials and methods

This study was approved by AgResearch Ltd, Grasslands AEC number 11079.

The test products used in this study are protected by a patent IIPONZ 581222.

12.3.1 Cows and treatments administered

Forty-eight clinically healthy cows with four functional quarters from Massey University *Agricultural Farm Services Dairy No 1* were used in this study. Cows with somatic cell counts below 200,000 cells/mL at 15 days before drying-off and below 300,000 cells/mL at 6 days before drying-off, no teat or palpable quarter abnormalities and negative Rapid Mastitis Test (RMT) were included. Cows were ranked according to ascending somatic cell counts then animals were assigned to treatment groups blocked on milk production. Allocation was for a one-way design in randomised blocks (n=14) using the randomisation function in Excel (Microsoft Office; *Microsoft Corporation*, USA). Allocation was as follows. Group 1 cows (n=14), were treated with an internal teat sealant formulation 1 containing 0.5% chlorhexidine (ATS1; *Bomac Ltd*, Auckland, New Zealand); Group 2 cows (n=14), were treated with internal teat sealant formulation 2 containing 0.5% chlorhexidine (ATS2; *Bomac Ltd*); and Group 3 cows (n=14), were treated with commercial teat sealant (Teatseal™, *Pfizer Animal Health*, Auckland). Every seventh animal was an untreated control; Group 4 (n=6). Three cows were removed from the study due to abortion (n=1; group 3), down cow (n=1; group 2), and loss of follow-up (n=1; group 2). Cows were run as a single separate mob for the duration of the study.

Treatments were administered intramammary (Figures 12.1 and 12.2) using the partial insertion technique after cleaning and disinfecting all of the teats with teat wipes (*Bomac Teat wipes*, *Bomac Ltd*), including wiping of the negative controls.

12.3.2 Procedures

Milk sampling. Quarter milk samples were taken aseptically four days before drying-off, on the day of drying-off, on the day of calving and four days later. Additional quarter milk samples were taken on the day of calving and four days immediately following the aseptic sampling to determine somatic cell counts. Milk samples were cultured and typed at the *Microbiology Laboratory* of the Institute of Veterinary, Animal, and Biomedical Sciences (IVABS), Massey University. The somatic cell count was determined instrumentally at SAITL Dairy Laboratories (Hamilton, New Zealand) using a Fossomatic 5000 (*Foss*, Hilleroed, Denmark) counter.

Bacterial challenge. Cows were challenged with a *Streptococcus uberis* S210 strain at 2 and 4 days after drying off. The preparation of the bacterial challenge broth has been

previously described (Fernandez 2007). In brief, a previously frozen, then thawed isolate of *Strep. uberis* S210 was streaked onto 5% sheep blood agar plates (*Fort Richard Laboratories Ltd*, Auckland, New Zealand) and incubated for 48 hours at $37\pm 2^{\circ}\text{C}$ under CO_2 -enriched aerobic conditions. Colonies that grew were harvested, re-suspended in normal saline (0.9% w/v NaCl) and inoculated into cell culture flasks with vent caps (*Corning Costar Corporation*, Cambridge, MA, USA), containing 20 ml of 5% blood agar medium. After incubation, colonies were harvested by scraping the surface of the media using normal saline and sterile glass beads. The turbidity of the new bacterial suspension was adjusted to a McFarlane turbidity standard of 0.5 (*Remel*, Lenexa, Kansas, USA) by adding normal saline. Approximately 40 ml of such prepared broth was dispensed into the plastic containers used for dipping the teats. Teats were dipped in the broth for 1-2 seconds by one person blinded to treatment. New broth was prepared for each day of challenge. The concentration of colony-forming units per mL of a *Strep. uberis* S210 strain in the broth was determined after infusion on a retained portion of the challenge broth and it was $\sim 1 \times 10^8$ on both occasions.

Clinical examination and treatment of clinical cases. Udders and teats were visually examined and palpated daily to check for signs of mastitis until 34 days after drying off, with the exception of Days 1 and 3, by a person blinded to treatment allocation. This period was the palpation period. Each quarter was subjectively judged as “mastitic” (score ≥ 3) or non-mastitic (score ≤ 2) according to standardised criteria developed by KRP (Table 12.1).

Quarters affected by clinical mastitis after challenge were sampled for microbial culture before treatment was administered to them. Any quarter/s with mastitis were then treated according to the clinical presentation. For the treatment of affected quarter/s *Ubro Yellow* (*Boehringer Ingelheim NZ*, Auckland, New Zealand; containing penethamate hydriodide, dihydrostreptomycin, framycetin and prednisolone) was administered once daily for three days after complete milking-out of the affected quarter/s. This treatment regime was used when up to three quarters in the same cow were affected. When four quarters were affected in the same cow, *Mamyzin* (*Boehringer Ingelheim NZ*; containing penethamate hydriodide) was administered intramuscularly once daily with 10g on the first day and 5 g on the two subsequent days. Thereafter, quarters were observed daily and treated as required, but any subsequent episode of mastitis in the same quarter was not included in the statistical analysis.

The incidence of clinical mastitis during the palpation period was calculated as the number of quarters affected by clinical mastitis divided by the number of quarters in a group. The incidence of clinical mastitis caused by the challenge organism during the palpation period was calculated as ‘the number of quarters affected by clinical mastitis caused by *Strep. uberis* of the total number of quarters in a group’, expressed as a percentage.

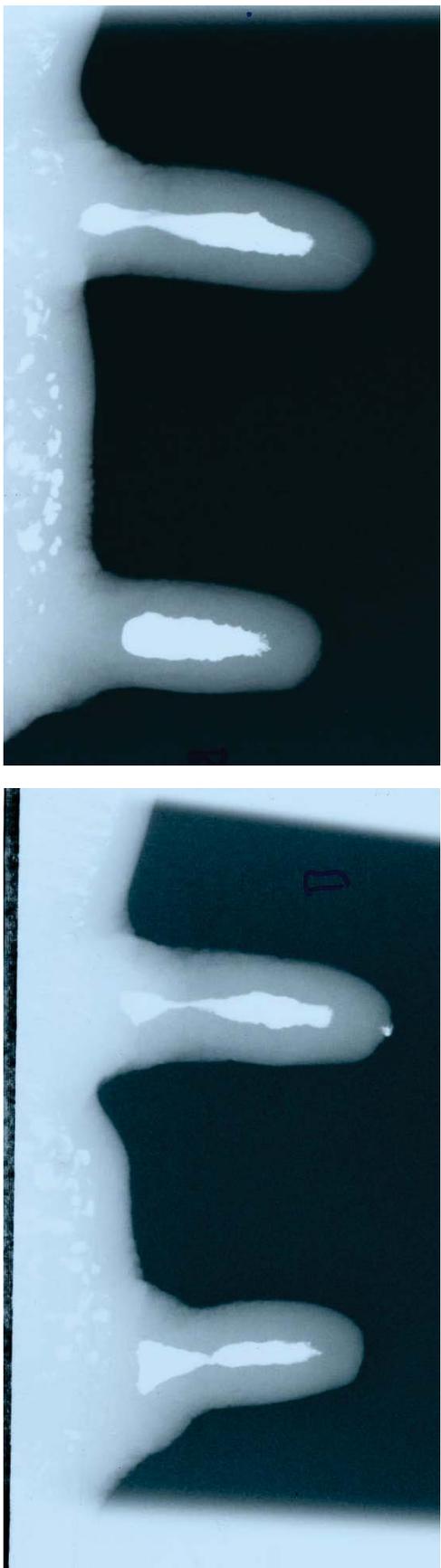


Figure 12.1 X-ray picture of the location of the internal teat sealant post treatment with the sealant showing as a bright white area within the teat cavity



Figure 12.2 X-raying of the teats after treatment in order to evaluate the position of the teat sealant as shown in Figure 12.1

Table 12.1. Quarter and teat examination and palpation scores and description (criteria developed by KRP)

Score	Description
0	No evidence of irritation, soreness, redness or swelling of the quarter. No stripping of quarter/s.
1	No or virtually no evidence of irritation, soreness, redness or very slight swelling of the quarter. No stripping of quarter/s.
2	Evidence of irritation or soreness of a minor intensity or slight redness or swelling, likely to originate from residual milk in the quarter. No stripping of quarter/s.
3	Evidence of irritation or soreness of a moderate intensity or moderate redness or swelling of the quarter. Secretion contains small cloths and flecks.
4	Evidence of irritation or soreness of a severe intensity or severe redness (beet redness) or severe swelling of the quarter. The secretion contains large clots and flecks.
5	Evidence of severe irritation or soreness, severe swelling or redness, associated with generally sick animal. The secretion contains large clots and flecks.

Culturing of samples. Microbial culture was carried out by spreading 10 µL of milk from a single gland onto a quadrant of a 5% sheep blood agar plate, which was incubated for up to 72 h at 35-37°C in aerobic conditions (Hogan *et al.* 1999). Results from culture were also assessed according to guidelines from the National Mastitis Council:

- a) Growth of more than two colony types on a quadrant was reported as a contaminated sample.
- b) No, one or two colonies growing on a quadrant was reported as an uninfected sample.
- c) More than two colonies, but less than three colony types growing on the quadrant was reported as infected sample and the predominant colony type was isolated and identified.

Identification of the cultured isolates was carried out by an assessment of colony morphology, Gram stain reaction and a number of biochemical tests. Bacilli were classified as Gram positive or Gram negative bacilli, with no further testing. *Corynebacterium bovis* isolates were identified based on their cultural characteristics and colony morphology. Gram-positive, catalase-positive organisms were categorized as either coagulase-positive or coagulase-negative staphylococci based on the results of a tube rabbit plasma coagulase test (*Remel*). Gram-positive and catalase-negative organisms were further tested for their ability to hydrolyse aesculin (*Fort Dodge*, Auckland, New Zealand). The aesculin-negative organisms were subjected to the Christie–Atkins–Munch–Petersen (CAMP; *Fort Dodge*) reaction and categorized as either CAMP-positive or CAMP-negative bacteria. Non-haemolytic, aesculin positive, Gram-positive cocci were further tested for their ability to grow in buffered azide glucose glycerol broth (BAGG; *Fort Dodge*) and ferment inulin (*Fort Dodge*). BAGG-positive inulin-fermenters were identified as *Strep. uberis*. All the other Gram-positive, catalase-negative cocci were identified as *Streptococcus* spp.

The presence of an intramammary infection in any quarter after calving was determined based on an assessment of the culture results. A gland was defined as being infected if there was a growth of ≥ 3 colony forming units per quadrant of any of the major mastitis-causing organisms (*i.e.* coagulase-positive staphylococci and streptococci) at a sampling and if a minor or uncommon or any combination of mastitis-causing organism/s was isolated at both samplings after calving. The prevalence of infection after calving was

evaluated independently of the infection status before drying-off (data not shown) and the incidence of clinical mastitis during the palpation period. Any quarters with contaminated samples or missing samples after calving were excluded from analysis unless their sample contained a major-mastitis causing organism.

Genotyping of isolates. In order to assess the genetic relatedness to the challenge strain of *Strep. uberis* isolates causing intramammary infections, those isolated during the palpation period were genetically characterised using Pulsed-Field-Gel Electrophoresis (PFGE) of *Sma*I DNA macrorestriction fragments.

*Sma*I chromosomal digestion and PFGE conditions were as previously described (McDougall *et al.* 2004), with the following modifications: The optical density of the bacterial suspension was measured and adjusted in a spectrophotometer (*Unicam Limited*, Cambridge, UK). An aliquot was centrifuged, the supernatant removed twice and the precipitate re-suspended in cold cell suspension buffer and re-centrifuged forming a suspension. The suspension was mixed with melted low-melt agarose (*Bio-Rad Laboratories*, Hercules, CA, USA) and an aliquot of the mixture was placed into a plug moulding well (*Bio-Rad Laboratories*). Once solidified, plugs were suspended in a buffer containing lysozyme (*Roche Diagnostics*, Indianapolis, IN, USA) and incubated. Following incubation, the lysis buffer was removed and an ESP buffer containing proteinase K (*Roche Diagnostics*) was added and re-incubated. The plugs were then washed and stored. Each plug was prepared and exposed to cutting buffer with *Sma*I [New England] (*Biolab*, Auckland, New Zealand).

The macro-restriction fragments were separated by PFGE with a pulse angle of 120°, a gradient of 6V/cm, and a 23-h run in a contour-clamped homogenous electric field apparatus (CHEF Mapper machine, *Bio-Rad Laboratories*). 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, briefly rinsed 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*), with a maximum position tolerance of 1%. Bands were described by their sizes in kilobases (kb) and were identified from the highest molecular weight band to the lowest.

The DNA banding patterns of the tested strains were visually compared with the pattern of the challenge strain. Isolates were defined as indistinguishable from the challenge strain (*i.e.* the same banding pattern), closely related (up to three bands difference) or unrelated when there were more than 3 bands different (Tenover *et al.* 1995; McDougall *et al.* 2004).

12.3.3 Statistical analysis

All analyses were undertaken using the statistical software SAS (Statistical Analysis System, *SAS Institute Inc.*, Cary, NC, USA 2003) version 9.1. Milk samples for culturing, somatic cell count and palpation scores were taken at the quarter level.

The length of the dry period was analysed using the MIXED procedure with a linear model that considered the fixed effect of treatment group (ATS1, ATS2, commercial teat sealant, Control). The model outputs were the least square means and their standard errors.

Udder palpation scores were analysed using the MIXED procedure with a mixed linear model that included the fixed effect of treatment and random effect of a cow. The least square means of the palpation scores per group and their standard errors were the model outputs and used for multiple comparisons.

Records of clinical mastitis for each quarter during the 34 day palpation period were analysed using the GLIMMIX procedure with a logistic regression model that included the fixed effect of treatment (ATS1, ATS2, commercial teat sealant and untreated controls) and the random effect of a cow. The variable had a binomial distribution (1=quarter with clinical mastitis and 0=healthy quarter). Mean and 95% Confidence Interval (95% CI) of the incidence of clinical mastitis for each treatment were obtained after back-transformed to the binomial scale.

The prevalence of infected quarters after calving was also analysed using the GLIMMIX procedure with a logistic regression model including the fixed effects of treatment (ATS1, ATS2, commercial teat sealant and untreated controls) and random effect of a cow. Least square means and standard errors of the prevalence of infected or non-infected quarters at each sampling point were back-transformed and are presented as means and their standard errors. The prevalence of contaminated quarters was not different between the groups and they were excluded from the analysis.

Somatic cell score was calculated as $SCS = \text{Log}(SCC/1000)$ and analysed using the MIXED procedure with a mixed model for repeated measures, considering the fixed effect of treatment (ATS1, ATS2, commercial teat sealant and untreated controls), day after calving and their interaction and the random effect of quarter nested within cow. The least squares means and standard errors of SCS for each treatment group and day of measurement were obtained.

The level of significance was set at $P < 0.05$.

12.4 Results

12.4.1 Length of dry period

Means of the dry period lengths were similar across groups (Table 12.2).

Table 12.2. Means and standard errors of the lengths of the dry period in days per group

Treatment group	Dry period \pm SE
ATS 1	60.1 \pm 3.2
ATS 2	62.5 \pm 3.2
Commercial teat sealant	55.3 \pm 3.2
Control	66.8 \pm 4.7

12.4.2 Palpation scores

The average palpation scores (Table 12.1) per group are presented in Table 12.3. The score of the untreated quarters was significantly higher than that in all treated groups.

Table 12.3. Palpation scores in the first 34 days after drying-off adjusted for the random effect of an individual cow

Treatment group	Palpation scores \pm SE
ATS 1	0.08 \pm 0.05 ^x
ATS 2	0.14 \pm 0.05 ^x
Commercial teat sealant	0.20 \pm 0.05 ^x
Control	0.41 \pm 0.07 ^y

Superscripts indicate a significant difference ($P < 0.05$) within the column

The daily occurrence of quarters affected by clinical mastitis varied between the groups. Quarters of cows from the group treated with ATS1 were observed with clinical mastitis

on Days 13 (1/56), 20 (1/56) and 23 (1/56). For the group treated with ATS2, quarters with mastitis were observed on Days 6 (1/56), 9 (1/56), 11 (1/56), 12 (1/56) and 19 (4/56). For the group treated with commercial teat sealant, quarters with mastitis were observed on Days 5 (1/56), 6 (1/56), 7 (1/56) and 25 (1/56). Finally, for the untreated group, quarters with mastitis were observed on Days 6 (1/24), 8 (2/24), 9 (3/24), 10 (2/24), 11 (9/24), 12 (7/24) and 25 (1/24) (Table 12.4 and Figure 12.3).

Table 12.4. Effect of treatment on palpation scores in the first 34 days after drying-off

Score	Bomac ATS1		Bomac ATS2		Commercial teat sealant		Untreated		Total	
	No	%	No	%	No	%	No	%	No	%
0	1677	93.6	1599	89.2	1506	84.0	562	73.2	8906	87.0
1	89	5.0	152	8.5	220	12.3	128	16.7	727	9.6
2	23	1.3	33	1.8	62	3.5	53	6.9	176	2.8
3	1	0.1	7	0.4	0	0.0	23	3.0	23	0.5
4	2	0.1	1	0.1	4	0.2	2	0.3	4	0.2
5	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0

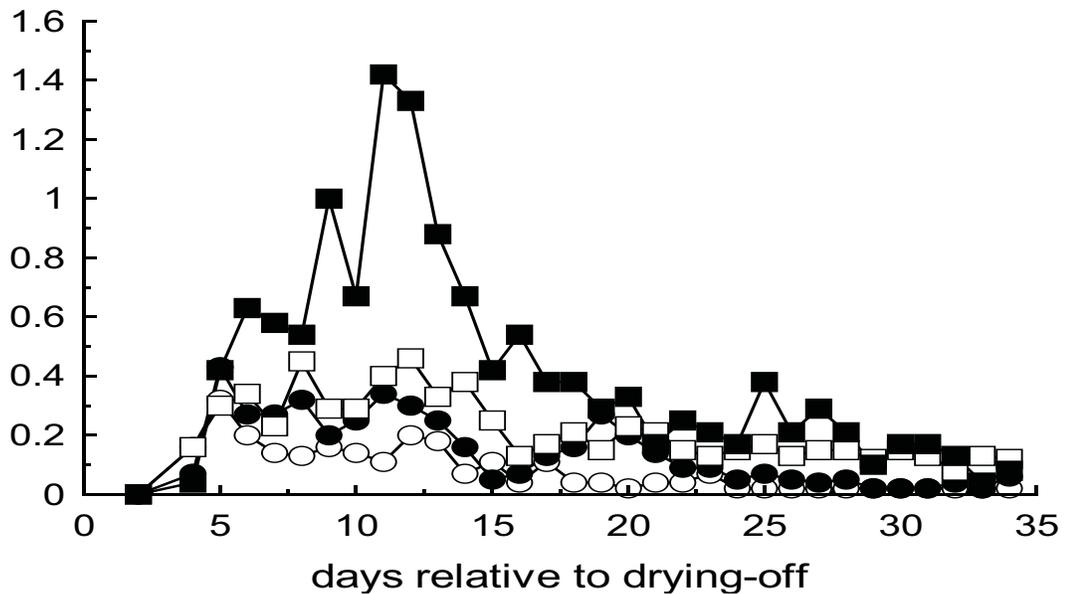


Figure 12.3. Average daily udder palpation score (-o- ATS 1; -●- ATS 2; -□- commercial teat sealant; -■- control)

12.4.3 Clinical mastitis

During the palpation period 21 quarters of 10 cows developed a clinical mastitis. The incidence of clinical mastitis during the palpation period in the negative controls was significantly higher than in all treated groups ($P < 0.05$; Table 12.5). All cows from the untreated control group developed mastitis in at least one quarter per cow. The challenge strain caused infection in 12 of 14 infected quarters of untreated controls. One cow of the group treated with ATS 2 developed sterile mastitis in all four quarters. In cows treated with ATS1 and the commercial teat sealant, one cow from each group developed mastitis caused by the challenge strain in one quarter.

Table 12.5. Incidence of clinical mastitis during the palpation period by treatment

Group	No. of quarters	Clinical mastitis	<i>Strep. uberis</i>	Incidence of CM Percent (95% CI)
ATS 1	56	1	1	1.4 (0.1-12.8) ^x
ATS 2	56	4	0	3.4 (0.6-17.3) ^x
Commercial teat sealant	56	2	1	1.4 (0.1-12.8) ^x
Control	24	14	12	61.8 (22.9-89.8) ^y

Superscripts indicate a significant difference ($P < 0.05$) within the column

12.4.4 Genotyping isolates of *Streptococcus uberis* from clinical cases

Two of the *Strep. uberis* isolates differed in one band and were therefore closely related to the challenge strain and the other 12 isolates were identical to the challenge strain. Hence, all infections caused by *Strep. uberis* were confirmed to be caused by the challenge strain.

12.4.5 Intramammary infection

The number of quarters infected after calving varied between the treatment groups (Table 12.6). These results have probably been influenced by the occurrence of clinical mastitis during the palpation period since affected quarters were treated during the palpation period and thus had a lower likelihood of being culture positive. Only two of the quarters treated in untreated controls during the palpation period were still infected with *Strep. uberis* after calving.

Table 12.6. Prevalence of quarters with intramammary infection after calving

Treatment group	No. excluded quarters	No. included quarters	Least square mean (95% CI)
ATS 1	16	36	22.9 (8.4-48.9)
ATS 2	14	39	6.2 (1.3-24)
Commercial teat sealant	23	29	17.2 (4.9-45.4)
Control	6	18	22.2 (5-61)

No difference in the prevalence of intramammary infections at calving was observed between the groups ($P>0.05$; Table 12.7).

Table 12.7. Summary of the culture results in per cent (and numbers) after calving (D0 - day of calving; D4 - day 4 after calving) for all sampled quarters (including those treated for clinical mastitis during the palpation period)

Group		No Growth	<i>Streptococcus uberis</i>	<i>Bacillus</i>	<i>Staphylococcus</i>	<i>Corynebacterium</i>	Contaminated
ATS 1	D0	67.3 (35)	0.0 (0)	15.4 (8)	5.8 (3)	0.0 (0)	11.5 (6)
	D4	59.6 (28)	0.0 (0)	19.1 (9)	4.3 (2)	0.0 (0)	17 (8)
ATS2	D0	76.9 (40)	0 (0)	9.6 (5)	1.9 (1)	1.9 (1)	9.6 (5)
	D4	77.1 (37)	0.0 (0)	10.4 (5)	2.1 (1)	0.0 (0)	10.4 (5)
Commercial teat sealant	D0	61.5 (32)	0.0 (0)	13.5 (7)	9.6 (5)	0.0 (0)	15.4 (8)
	D4	37.5 (18)	0.0 (0)	18.8 (9)	2.1 (1)	0.0 (0)	41.7 (20)
Control	D0	70.8 (17)	4.2 (1)	8.3 (2)	0.0 (0)	0.0 (0)	16.7 (4)
	D4	66.7 (16)	4.2 (1)	8.3 (2)	4.2 (1)	0.0 (0)	16.7 (4)
Total	D0	68.9 (124)	0.6 (1)	12.2 (22)	5.0 (9)	0.6 (1)	12.8 (23)
	D4	59.3 (99)	0.6 (1)	15.0 (25)	3.0 (5)		

12.4.6 Somatic cells

The test products showed no evidence of causing irritation as measured by daily udder and teat examinations during the palpation period and somatic cell scores after calving (Table 12.8). There were significant differences in the means of the somatic cell scores of milk from cows treated with the test products, the group treated with the commercial teat seal and the control group on Day 0 after calving and the negative control group on Day 4 after calving (Table 12.8).

Table 12.8. Means and their standard errors of the somatic cell scores (log of the somatic cell count divided by 1,000) among groups after calving

Group	No. of quarters	Average ± SE	No. of quarters	Average ± SE
		Day 0		Day 4
ATS 1	51	6.01 ± 0.19 ^x	52	4.40 ± 0.19 ^x
ATS 2	52	6.46 ± 0.19 ^x	48	4.70 ± 0.19 ^x
Commercial teat sealant	49	6.98 ± 0.19 ^y	52	4.83 ± 0.19 ^x
Control	24	6.93 ± 0.27 ^y	20	5.12 ± 0.27 ^y

Superscripts indicate a significant difference (P<0.05) within the column

12.5 Discussion

This study investigated the efficacy of two developmental internal teat sealants containing chlorhexidine against an experimental microbial challenge with *Strep. uberis* and evaluated the irritation caused when they were administered to dairy cattle at drying-off. The results demonstrated significant protection from intramammary infection that matched the existing commercial sealant and was significantly better than the untreated control quarters. Additionally, the developmental products did not cause irritation.

The study was designed to demonstrate the difference between the developmental products and no treatment and no difference between the developmental products and the commercial internal teat sealant. Therefore, the numbers of treated cows was relatively small. The results confirmed protection against the experimental challenge with *Strep. uberis* in quarters treated with the developmental products. The bacterial challenge in the present study resulted in nearly 46% of unprotected quarters being affected by clinical mastitis during the palpation period. This was similar to the success rate of 42%

in causing clinical mastitis in unprotected quarters previously seen using the same challenge protocol and organism (Fernandez 2007) and was higher than 32% in another study (Petrovski *et al.* 2011). The strain characteristic of high virulence in the early dry period or the high numbers of bacteria used in the challenge could explain the high rate of infection achieved by it.

Not all cases of clinical mastitis during the palpation period were confirmed to be due to the challenge strain. However, all the *Strep. uberis* isolates were identical (Zadoks *et al.* 2000; Zadoks and Schukken 2006) to the challenge strain, indicating the challenge organism caused these infections. Samples yielding different pathogens may represent background cases of mastitis that occur during the early dry period, or alternatively, the challenge procedure may have rendered the quarters more susceptible to new intramammary infections with field organisms. The incidence of naturally occurring clinical mastitis during the dry period in New Zealand is difficult to predict. It is usually very low, being less than 1-2%. In a study involving seven organic herds, only 1.6% of quarters had clinical mastitis (Berry and Hillerton 2002). Natural challenge in the New Zealand pastoral system causes an infection rate in unprotected quarters of approximately 16% (Woolford *et al.* 1998) compared to that seen in the present study of just over 22%. No significant difference in the prevalence of intramammary infection after calving was found in the present study. This may be due to the fact that many of the quarters that did not receive a treatment at drying off succumbed to mastitis during the palpation period were treated then for clinical mastitis. Therefore, the prevalence of intramammary infections after calving observed in the study is applicable for the study population, but it should not be extrapolated to the external population.

The test products ATS1 and ATS2 did not cause detectable irritation in the treated cows. Cows treated with the test products demonstrated significantly lower somatic cell scores at both test days after calving compared to the non-treated controls. The somatic cell count at the first milking was also significantly lower than in milk from cows in the group treated with the commercial teat sealant after calving.

The average length of the dry period was similar between the groups. Thus, the observed differences cannot be a result of changes in the intramammary infection rate associated with longer dry periods (Natzke *et al.* 1975; Rindsig *et al.* 1978; Berry and Hillerton 2007). The lower somatic cell count in the cows treated with the test products may be

explained by a lower irritancy of these products, or better protection against experimental and natural bacterial challenge. The better protection against invading pathogens appears to have reduced the infection challenge to the mammary gland resulting in less mastitis and a smaller influx of white blood cells as demonstrated by the somatic cell score. The sample size limited the power of this study to detect significant differences in infection status or protection at calving. However, somatic cell counts, which are known to be highly related to mastitis infection, were significantly lower at the first post-calving sampling in groups treated with the two test products, suggesting that protection against infection in the dry period was superior. This observation requires re-assessment in a study with more power.

12.6 Acknowledgments

The authors thank *Bomac Ltd* for the financial support; Jeremy Lind from *Invoco AgResearch*; the staff from *Agricultural Farm Services*, Massey University for their involvement in the animal phase; Hamish Mack and Rebecca Pattison from the *Microbiology Section*, IVABS, Massey University; and staff at *SAITL Dairy Laboratories* for their involvement in the analytical phase of the study. KR Petrovski's position at Massey University is supported by *Bomac a company of Bayer Ltd*.

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**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Kiro R Petrovski

Name/Title of Principal Supervisor: Prof Norman B Williamson

Name of Published Paper: A preliminary evaluation of the efficacy of two novel internal teat sealant formulations against bacterial challenge in the early dry period

In which Chapter is the Published Work: Chapter 12

What percentage of the Published Work was contributed by the candidate: 70%



Candidate's Signature

11 July 2011

Date



Principal Supervisor's signature

14/7/2011

Date

Chapter 13

Efficacy of a novel internal dry period teat sealant containing 0.5% chlorhexidine against experimental challenge with *Streptococcus uberis* in dairy cattle

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Publication details:

Petrovski KR, Caicedo-Caldas A, Williamson NB, Lopez-Villalobos N, Grinberg A, Parkinson TJ, Tucker IG. Efficacy of a novel internal dry period teat sealant containing 0.5% chlorhexidine against experimental challenge with *Streptococcus uberis* in dairy cattle. *Journal of Dairy Science* 94, 3366-75, 2011

13. Efficacy of a novel internal dry period teat sealant containing 0.5% chlorhexidine against experimental challenge with *Streptococcus uberis* in dairy cattle

13.1 Abstract

AIMS: The incidence of clinical mastitis and infection status at calving was assessed in quarters treated with one of two internal teat sealants at the time of dry off.

METHODS: Two contralateral quarters per cow (n=63 cows) were treated with a sealant that contained 0.5% chlorhexidine; the other quarters were treated with a commercial teat sealant. Ten cows were untreated (controls). On days 2, 4 and 16 after dry off, cows were challenged with *Streptococcus uberis* S210 strain. Cows were examined daily for 34 days after drying off and cases of clinical mastitis were recorded. Milk samples were collected for culture from any quarters that developed clinical mastitis during the first 34 days after drying-off and from all quarters on Days -5 and 0 relative to treatment and at the 1st and 20th milking after calving.

RESULTS: The incidence of clinical mastitis during the examination period was lower in treated quarters (n=7/252; 1.5%; lower for those treated with chlorhexidine-containing teat sealant n=3/126; 1.2%) than in untreated quarters (n=13/40; 26.8%). The protection against intramammary infection after calving, adjusted for the effect of cow, was higher in quarters treated with the novel teat sealant (89/105; 15.2% 95% CI=9.6-23.4) than in those treated with the commercial teat sealant (71/104; 31.7% 95% CI=23.5-41.3) and untreated controls (6/28; 78.6% 95% CI=59.8-90.0), respectively.

CONCLUSIONS: Quarters treated with teat sealants were less likely to have an intramammary infection after calving and had a lower incidence of clinical mastitis during the early dry period than untreated controls in this challenge study.

KEY WORDS: internal teat sealant, challenge, dry period, *Streptococcus uberis*

BAGG - Buffered Azide Glucose Glycerol broth, CAMP - Christie-Atkins-Munch-Petersen test, CI - Confidence Interval, DCT - Dry Cow Therapy (with antimicrobials), LF - Left Front, RR - Rear Right

13.2 Introduction

Intramammary infections during the dry period are prevented by minimising bacterial challenge from the environment and maximising and supplementing the defence mechanisms of the mammary gland (Bradley and Green 2004).

Antibiotic dry cow therapy (DCT) is a means of preventing new infections during the dry period and of eliminating existing subclinical infections. Treatment with antimicrobials at drying off risks the development of resistant strains of bacteria and violative antibacterial residues in milk after calving. To avoid these risks, artificial teat sealants were developed to prevent new intramammary infections (Meaney 1977; Woolford *et al.* 1998). Teats which become 'closed' by the keratin plug or an artificial seal after drying off are less likely to become infected in the dry period (Woolford *et al.* 1998; Berry and Hillerton 2002b; Huxley *et al.* 2002). The barrier formed by a sealant occurs faster than without treatment thus reducing the entry of mastitis-causing organisms into the gland while a keratin plug forms.

New Zealand's pasture-based seasonal dairy system is associated with some specific problems for the management of the dry period. The length of the dry period is variable and in many cases cows are dried-off as dictated by pasture growth and feed availability. The rate of new intramammary infections is related to the length of the dry period. Longer dry periods have been associated with an increase in the incidence of intramammary infections (Natzke *et al.* 1975; Rindsig *et al.* 1978; Bradley and Green 2004; Berry and Hillerton 2007; Laven 2008). This may relate to the duration of action of the DCT, as the concentration of antibiotic falls and the protective role against infection challenge is diminished (Bradley and Green 2000; Sanford *et al.* 2006; Berry and Hillerton 2007). The efficacy of internal teat sealants appears unaffected by the length of the dry period when used alone or in combination with DCT (Woolford *et al.* 1998; Huxley *et al.* 2002; Berry and Hillerton 2007). Since the prediction of calving date in New Zealand is often not reliable and the infection status of cows is unknown, the best mastitis protection is expected from a combined use of DCT and internal teat sealant (Bradley and Green 2004). For known uninfected quarters the use of internal teat sealant alone has been advocated (Woolford *et al.* 1998; Bradley and Green 2004).

The use of internal teat sealants presents the risk of introducing new intramammary infections during their administration. This risk could potentially be reduced if an

antimicrobial compound was incorporated into the sealant (Ryan *et al.* 1998; Crispie *et al.* 2004a) if it possesses a suitable spectrum of activity.

This study compared the efficacy of a teat sealant containing chlorhexidine with a commercial teat sealant not containing an antimicrobial agent and with untreated controls. Treatments were administered at drying-off to healthy dairy cows which were subsequently challenged with a known strain of *Streptococcus uberis*. Chlorhexidine was used because of its activity against most Gram-positive bacteria of importance in New Zealand and other infectious organisms, including some Gram-negative bacteria when it is at higher concentrations (Heit and Riviere 2009). The null hypothesis tested was that chlorhexidine-containing teat sealant would not affect the incidence of clinical mastitis in the dry period nor the prevalence of intramammary infections after calving.

13.3 Materials and methods

This study was approved by Kaiawhina Animal Ethics Committee (AEC 005/09).

13.3.1.1 Animals

Seventy-three cows less than 8 years old from Massey University *Agricultural Farm Services* Dairy Number 4 (Palmerston North, New Zealand) with negative California Mastitis Test (CMT) and <200,000 cells/mL 9 days before drying-off were used in the present study. Cows in this spring calving dairy herd were grazed on a ryegrass-white clover pasture supplemented as needed with pasture silage and milked through a 50 stall rotary dairy shed twice daily during lactation. The experimental unit was the quarter. Sixty-three cows were allocated as treatment cows (treated group) and ten were untreated controls (untreated group). Treated cows had a front and a contra-lateral rear quarter treated with the novel chlorhexidine-containing teat sealant and the remaining two quarters treated with a commercial teat sealant. The treatment was alternated between the cows. Cows were randomised on somatic cell count using the block randomisation seed option of GenStat software (version 9.1; *VSN International*, Hemel Hempstead, UK). Five cows failed to complete the study due to abortion (1; untreated), traumatic injury resulting in death (1; treated), clinical milk fever resulting in death (1; treated) and being culled as non-pregnant (2; treated) leaving data from 68 cows for analysis of intramammary infection status at calving.

13.3.2 Treatment products and treatment administration

Two treatment products were used in this study:

1. Bomac ATS, containing bismuth subnitrate 65% and chlorhexidine 0.5% (*Bomac Ltd*, Auckland, New Zealand) referred to as chlorhexidine-containing teat sealant.
2. Teatseal™, containing bismuth subnitrate 65% (*Pfizer Animal Health*, Auckland) as a positive control referred to as commercial teat sealant.

Treatments were administered within two hours after the last milking for the 2008/09 season using the partial insertion technique (Boddie and Nickerson 1986). Before treatment, teats of all cows (including untreated controls) were cleaned and disinfected with alcohol-based teat wipes (Bomac Teat wipes, *Bomac Ltd*). No massage of the teats was carried out after treatment administration but the teats of all cows (including untreated controls) were sprayed with an iodine-based teat spray (TeatGuard Plus, *Ecolab Ltd*, Hamilton, New Zealand) following label recommendations.

13.3.3 Procedures

Duplicate quarter-milk samples were collected aseptically 5 days before drying-off, on the day of drying-off and at the 1st and 20th milking after calving. All milk samples were cultured following the National Mastitis Council Guidelines (Hogan *et al.*, 1999) at the *Microbiology Laboratory* of the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University.

All cows were challenged twice, 2 and 4 days after drying-off, by dipping the teat barrel in the challenge broth for 1-2 seconds by a single person blinded to treatment. The concentration in the broth of colony-forming units of a *Strep. uberis* S210 strain on Days 2, 4 and 16 after treatment is shown in Table 13.1. Challenges were carried out in different facilities from the normal milking shed to avoid the milk let-down reflex. Separate containers were used to dip the LF (left front) and RR (rear right) quarters to those which were used for the other two quarters. In this way there was no possibility of cross-contamination between different products; nor was the blinding of the trial compromised. Each cow was dipped with two new challenge broths. A new broth was prepared for each day of challenge (Table 13. 1).

Table 13.1. The concentration of colony-forming units of a *Streptococcus uberis* S210 strain per millilitre in the challenge broth at different challenge days

Day after treatment	Concentration
2	7.7 x 10 ⁸
4	5.4 x 10 ⁷
16	2.3 x 10 ⁷

The challenge broth was prepared by thawing the isolate, streaking onto blood agar plates (*Fort Richard Laboratories Ltd*, Auckland, New Zealand), incubation at 37±2°C under CO₂-enriched conditions, harvesting colonies from the plates using cotton swabs (*Fort Richard Laboratories Ltd*) and suspending them in normal saline (0.9% w/v NaCl). The turbidity was adjusted to a McFarland turbidity standard of 0.5 (*Remel*, Lenexa, Kansas, USA) by adding normal saline.

Udders were visually examined and palpated daily until 34 days after drying-off, with the exception of Days 1 and 3, by a person blinded to treatment allocation. This was the defined palpation period. Quarters were observed and palpated for the presence of clinical signs consistent with mastitis. Each quarter was subjectively judged as “mastitic” (score ≥3) or non-mastitic (score ≤2) according to standardised criteria developed by KRP (Table 13.2). All examinations were carried out by a single veterinarian blinded to treatment.

Table 13.2. Quarter and teat examination and palpation scores and description (developed by KRP)

Score	Description
0	No evidence of irritation, soreness, redness or swelling of the quarter. No stripping of quarter/s.
1	No or virtually no evidence of irritation, soreness, redness or very slight swelling of the quarter. No stripping of quarter/s.
2	Evidence of irritation or soreness of a minor intensity or slight redness or swelling, likely to originate from residual milk in the quarter. No stripping of quarter/s.
3	Evidence of irritation or soreness of a moderate intensity or moderate redness or swelling of the quarter. Secretion contains small clots and flecks.
4	Evidence of irritation or soreness of a severe intensity or severe redness (beet redness) or severe swelling of the quarter. The secretion contains large clots and flecks.
5	Evidence of severe irritation or soreness, severe swelling or redness, associated with generally sick animal. The secretion contains large clots and flecks.

Quarters affected by clinical mastitis were sampled for microbial culture before treatment was administered to them. Microbial culture was carried out by spreading 10 µL of milk

from a single quarter onto a quarter of a 5% sheep blood agar plate, which was incubated for up to 72 h at 35-37°C in aerobic conditions (Hogan *et al.*, 1999). Any mastitic quarter/s were then treated according to the clinical presentation. For the treatment of affected quarter/s Ubro Yellow (*Boehringer Ingelheim NZ*, Auckland, New Zealand; containing penethamate hydriodide, dihydrostreptomycin, framycetin and prednisolone) was administered once daily for three days after complete milking-out of the affected quarter/s. This treatment regime was used when up to three quarters in the same cow were affected. When four quarters were affected in the same cow, Mamyzin (*Boehringer Ingelheim NZ*; containing penethamate hydriodide) was administered intramuscularly once daily with 10g on the first day and 5 g on the two subsequent days. Thereafter, quarters were observed daily and treated as required, but any subsequent episode of mastitis in the same quarter was not included in the statistical analysis.

The incidence of clinical mastitis during the palpation period was calculated as the proportion of quarters affected by clinical mastitis from the total number of quarters in a group. The incidence of clinical mastitis caused by the challenge organism during the palpation period was calculated as the percentage of quarters affected by clinical mastitis caused by *Strep. uberis* from the total number of quarters in a group. Whenever *Strep. uberis* was isolated it was assumed it was the challenge strain. Previous work by this group confirmed using a highly discriminative method (Pulse-Field Gel Electrophoresis) that all clinical cases during the dry period caused by *Strep. uberis* were identical to the challenge strain (Chapter 12).

Results from culture were assessed according to guidelines from the National Mastitis Council (Hogan *et al.* 1999):

- a) Growth of three or more colony types on a quadrant was reported as a contaminated sample.
- b) One or two colonies growing on the quadrant was reported as an uninfected sample.
- c) More than two colonies, but less than three colony types growing on the quadrant was reported as infected sample and the predominant colony type was isolated and identified.

Identification of the cultured isolates was carried out by an assessment of colony morphology, Gram stain reaction and a number of biochemical tests. Bacilli were classified as Gram positive or Gram negative bacilli, with no further testing.

Corynebacterium bovis isolates were identified based on their cultural characteristics and colony morphology. Gram-positive, catalase-positive organisms were categorised as either coagulase-positive or coagulase-negative staphylococci based on the results of a tube rabbit plasma coagulase test (*Remel*). Gram-positive and catalase-negative organisms were further tested for their ability to hydrolyse aesculin (*Fort Dodge*). The aesculin-negative organisms were subjected to the Christie–Atkins–Munch-Petersen (CAMP; *Fort Dodge*) reaction and categorised as either CAMP-positive or CAMP-negative bacteria. Non-haemolytic, aesculin positive, Gram-positive cocci were further tested for their ability to grow in buffered azide glucose glycerol broth (BAGG; *Fort Dodge*) and fermented inulin (*Fort Dodge*). BAGG-positive inulin-fermenters were identified as *Strep. uberis*. All the other Gram-positive, catalase-negative cocci were identified as *Streptococcus* spp.

The presence of an intramammary infection in any quarter after calving was determined based on an assessment of the culture results. A gland was defined as being infected if there was a growth of ≥ 3 colony forming units per quadrant of any of the major mastitis-causing organisms (*i.e.* coagulase-positive staphylococci, streptococci and Gram-negative rods) at a sampling and if a minor or uncommon or any combination of mastitis-causing organism/s was isolated at both samplings after calving. The prevalence of infection after calving was evaluated independently of the infection status pre-drying-off and the incidence of clinical mastitis during the palpation period. Any quarters with contaminated samples or missing samples after calving were excluded from analysis unless they were a sample containing a major-mastitis causing organism.

13.3.4 Statistical analysis

All analyses were undertaken using SAS (Statistical Analysis System, *SAS Institute Inc.*, Cary, NC, USA 2003) version 9.1.

Statistical differences between the cumulative percentages of quarters becoming infected in the treatment groups (chlorhexidine-containing teat sealant, commercial teat sealant and untreated controls) were analysed using survival analysis utilising the LIFETEST procedure.

Udder palpation scores for each quarter were analysed as categorical data by Fisher's exact test with respect to treatment (chlorhexidine-containing teat sealant, commercial teat sealant and untreated controls).

Records of clinical mastitis for each quarter during the 34-day palpation period were analysed using the GLIMMIX procedure with a logistic regression model that included the fixed effect of treatment (chlorhexidine-containing teat sealant, commercial teat sealant and untreated controls) and the random effect of a cow. The variable had a binomial distribution and analyses were carried out after the logit transformation. Least square means of incidence of clinical mastitis and their 95% Confidence Intervals (CIs) were obtained and back-transformed to the binomial scale. The same procedure was applied to compare the incidence of clinical mastitis in the treated *versus* untreated quarters during the palpation period.

The success in preventing intramammary infection measured as presence or absence of infection after calving was analysed with the GLIMMIX procedure. The logistic regression model included the fixed effect of treatment (chlorhexidine-containing teat sealant, commercial teat sealant and untreated controls) and random effect of a cow. Least square means and 95% confidence intervals (CIs) for intramammary infection after calving for each treatment were back-transformed and are presented as means and 95% CIs. The effect of the length of the dry period on the success of the prevention of intramammary infection after calving was not significant. Hence, it was not included in the final model for estimation of the success of the prevention of intramammary infection after calving. The prevalence of infected (positive on culture) quarters per group was also analysed using GLIMMIX procedure including the fixed effects of treatment (chlorhexidine-containing teat sealant, commercial teat sealant and untreated controls), sampling point and their interaction. Least square means and standard errors of the prevalence of infected or non-infected quarters at each sampling point were back-transformed and are presented as means and their standard errors. The prevalence of contaminated quarters was not different between the groups and they were excluded from the analysis.

The least squares means of lengths of dry periods and their 95% CIs and differences for the treatment groups were estimated using the MIXED procedure with a linear model that included the fixed effect of treatment (chlorhexidine-containing teat sealant, commercial teat sealant and untreated controls).

Table 13.3. Prevalence of infected or non-infected quarters in percent \pm standard errors among groups¹

Status	Day - 5			Day 0			Milking 1			Milking 20		
	ATS	TS	UC	ATS	TS	UC	ATS	TS	UC	ATS	TS	UC
Infected	31.40 \pm 0.04	33.90 \pm 0.04	28.21 \pm 0.12	32.80 \pm 0.04	35.48 \pm 0.03	27.50 \pm 0.12	3.48 \pm 0.24 ^b	22.12 \pm 0.05 ^a	23.33 \pm 0.18 ^a	12.96 \pm 0.08 ^b	20.00 \pm 0.06 ^b	54.54 \pm 0.12 ^a
	68.60 \pm 0.04	66.10 \pm 0.04	71.79 \pm 0.12	67.20 \pm 0.04	64.52 \pm 0.03	72.50 \pm 0.12	96.52 \pm 0.24 ^a	77.88 \pm 0.05 ^b	76.67 \pm 0.18 ^b	87.04 \pm 0.08 ^a	80.00 \pm 0.06 ^a	45.46 \pm 0.12 ^b

^{a-b}Values within rows and sampling points after calving having different superscript letters differ (P<0.05)

¹d -5 and 0 are days relative to drying off; milking 1 and milking 20 are the number of milkings after calving. ATS = treatment with a teat sealant containing bismuth subnitrate 65% and chlorhexidine 0.5% (*Bomac Laboratories Ltd*, Auckland, New Zealand); TS = treatment with Teatsal, containing 65% bismuth subnitrate (*Pfizer Animal Health*, Auckland), a positive control commercial teat sealant; UC = untreated controls.

13.4 Results

13.4.1 Dry period

The lengths of the dry periods were similar between the treated (103.1 days; 95% CI=98.0-108.3) and untreated (96.1 days; 95% CI=83.0-109.3; P=0.334) cows.

13.4.2 Udder palpation scores

Palpation results were available for all 73 cows (Table 13.4) for the 34 days after drying off, with the exception that one cow from the untreated control group had udder palpation carried out only until Day 17, after which the cow aborted and was excluded from the study. Four further cows (all treated) missed palpations on a total of six occasions. The effect of treatment on the palpation scores was significant (P<0.001).

Table 13.4. Effect of treatment on palpation scores in the first 34 days after drying-off

Score	Chlorhexidine-containing teat sealant		Commercial teat sealant		Untreated		Total	
	n	%	n	%	n	%	n	%
0	3930	92	3898	91.3	1078	83.4	8906	90.5
1	291	6.8	299	7	137	10.6	727	7.4
2	48	1.1	70	1.6	58	4.5	176	1.8
3	3	0.1	5	0.1	15	1.2	23	0.2
4	0	0	0	0	4	0.3	4	0
5	0	0	0	0	0	0	0	0

n – number of observations.

The frequency of palpation scores ≥ 3 was highest on Days 10, 16 and 19 for the chlorhexidine-containing teat sealant treated quarters and on Days 6, 16 and 19 for commercial teat sealant treated quarters (maximum value of 3/126 quarters on Day 19). The frequency of palpation scores ≥ 3 in untreated quarters was highest on Days 9, 10 and 14, (4/40 quarters on each occasion).

13.4.3.1 Clinical mastitis during the palpation period

During the palpation period, 20 quarters from 9 cows developed clinical mastitis. Fourteen of these mastitis cases were caused by *Strep. uberis* (Table 13.5). Quarters treated with chlorhexidine-containing teat sealant had 1.2% infected (95% CI=0.3-5.4) versus 26.8% in untreated quarters (95% CI 7.6-62.0; P<0.001).

Table 13.5. Distribution of cases of clinical mastitis (CM), the probability of a quarter being affected by CM (probability total) and probability of a quarter of being affected with CM caused by the challenge organism (probability challenge) in the first 34 days after drying-off

Group	Treatment group	Quarters affected by CM		Quarters with positive isolation		Probability total (% and 95% CI)	Probability challenge (% and 95% CI)
		n	%	n	%		
1	Chlorhexidine-containing teat sealant	3	2.4	1	0.8	1.2 (0.3-5.4)	0.7 (0.1-5.1)
1	Commercial teat sealant	4	3.2	1	0.8	1.8 (0.5-6.6)	0.7 (0.1-5.1)
2	Untreated	13	32.5	12	30.0	26.8 (7.6-62.0)	25.3 (8.6-55.0)

n – Number of quarters

The highest risk for the incidence of clinical mastitis during the palpation period was between 6 and 19 days after drying-off (Figure 13.1).

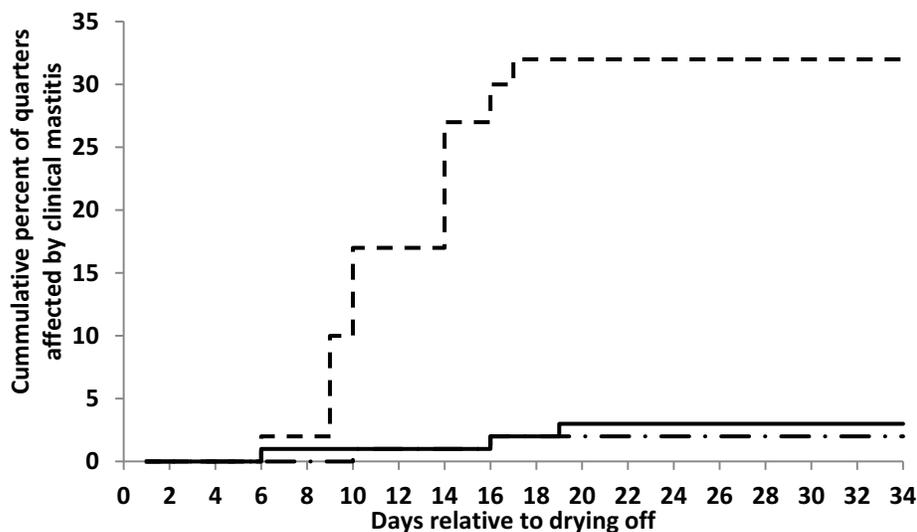


Figure 13.1. Survival analysis from treatment to incidence of clinical mastitis (Chlorhexidine-containing teat sealant - · -, Commercial teat sealant —, Untreated - - -) during the first 34 days after drying-off

13.4.4 Milk culture results

Thirteen contaminated samples, and 13 and 5 missing samples from the chlorhexidine-containing commercial teat sealant and untreated controls respectively were not available for analysis. The culture results are presented in Table 13.6. The distribution of mastitis-causing organisms was similar in the groups before drying-off, but differences were evident after calving.

A higher prevalence of major mastitis-causing organisms was detected in the untreated quarters and quarters treated with the commercial teat sealant than in quarters treated with the chlorhexidine-containing teat sealant. This was largely due to coagulase-positive staphylococci rather than the challenge organism. The prevalence of minor mastitis-causing organisms was 2.5% and 16.9% at the first milking and 10.2% and 13.6% at the 20th milking in quarters treated with the chlorhexidine-containing teat sealant and those treated with the commercial teat sealant, respectively. Quarters affected by clinical mastitis in the first 34 days after drying-off were treated at the time of diagnosis and signs of clinical mastitis subsided. Despite this, *Strep. uberis* was isolated from 7 quarters after calving; 5 from the untreated group and 1 of each treated with commercial or chlorhexidine-containing teat sealant.

Table 13.6. Summary of the culture results in percent (and numbers) among groups (D-5 and D0 ~ days relative to drying off; M1 and M20 ~ milkings after calving)

Organism	Chlorhexidine-containing teat sealant						Commercial teat sealant						Untreated					
	D-5	D0	M1	M20	D-5	D0	M1	M20	D-5	D0	M1	M20	D-5	D0	M1	M20		
CPS ¹	0.8 (1)	0.0 (0)	0.0 (0)	1.7 (2)	0.0 (0)	0.0 (0)	3.4 (4)	4.2 (5)	0.0 (0)	0.0 (0)	0.0 (0)	8.3 (3)	0.0 (0)	0.0 (0)	0.0 (0)	8.3 (3)		
<i>Streptococcus uberis</i>	0.0 (0)	0.0 (0)	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.8 (1)	0.0 (0)	2.5 (1)	0.0 (0)	15.6 (5)	0.0 (0)	0.0 (0)	0.0 (0)	15.6 (5)	0.0 (0)		
<i>Major total²</i>	0.8 (1)	0.0 (0)	0.8 (1)	1.7 (2)	0.0 (0)	0.0 (0)	4.2 (5)	4.2 (5)	2.5 (1)	0.0 (0)	15.6 (5)	8.3 (3)	0.0 (0)	0.0 (0)	15.6 (5)	8.3 (3)		
<i>Bacillus</i> spp	0.0 (0)	0.0 (0)	0.8 (1)	0.0 (0)	0.0 (0)	0.0 (0)	1.7 (2)	0.0 (0)	2.5 (1)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)		
Coagulase-negative staphylococci	23.4 (29)	32.5 (41)	0.0 (0)	7.6 (9)	26.6 (33)	34.9 (44)	10.2 (12)	11.0 (13)	22.5 (9)	25.0 (10)	0.0 (0)	25.0 (9)	25.0 (10)	0.0 (0)	0.0 (0)	25.0 (9)		
<i>C. bovis</i>	6.5 (8)	0.0 (0)	0.0 (0)	2.5 (3)	5.6 (7)	0.0 (0)	1.7 (2)	2.5 (3)	0.0 (0)	2.5 (1)	3.1 (1)	13.9 (5)	2.5 (1)	2.5 (1)	3.1 (1)	13.9 (5)		
Gram-negative rods	0.0 (0)	0.0 (0)	1.7 (2)	0.0 (0)	0.0 (0)	0.0 (0)	3.4 (4)	0.0 (0)	0.0 (0)	0.0 (0)	3.1 (1)	2.8 (1)	0.0 (0)	0.0 (0)	3.1 (1)	2.8 (1)		
<i>Minor total²</i>	29.8 (37)	32.5 (41)	2.5 (3)	10.2 (12)	32.3 (40)	34.9 (44)	16.9 (20)	13.6 (16)	25.0 (10)	27.5 (11)	6.3 (2)	41.7 (15)	25.0 (10)	27.5 (11)	6.3 (2)	41.7 (15)		
Contaminated	2.4 (3)	0.8 (1)	2.5 (3)	8.5 (10)	4.8 (6)	1.6 (2)	4.2 (5)	11 (13)	2.5 (1)	0.0 (0)	6.3 (2)	8.3 (3)	2.5 (1)	0.0 (0)	6.3 (2)	8.3 (3)		
No growth	66.9 (83)	66.7 (84)	94.1 (111)	79.7 (94)	62.9 (78)	63.5 (80)	74.6 (88)	71.2 (84)	70.0 (28)	72.5 (29)	71.9 (23)	41.7 (15)	70.0 (28)	72.5 (29)	71.9 (23)	41.7 (15)		
Number of sampled quarters	124	126	118	118	124	126	118	118	40	40	118	36	40	40	32	36		

¹Coagulase-positive staphylococci; ²CPS and *Streptococcus uberis* together; ³*Bacillus* spp, Coagulase-negative staphylococci, *C. bovis* and Gram-negative rods together.

The prevalence of intramammary infections after calving was significantly different between the groups (Table 13.7). It was highest in untreated quarters, followed by quarters treated with the commercial teat sealant. The lowest prevalence of intramammary infections after calving was in quarters treated with the chlorhexidine-containing teat sealant.

Table 13.7. Means and 95% confidence intervals of the quarter level infection rate after calving (Day 1 and 20 after calving)

Treatment	Mean	95% CI	Difference to chlorhexidine-containing teat sealant
Chlorhexidine-containing teat sealant	15.24	9.55-23.44	NA
Commercial teat sealant	31.73	23.52-41.26	0.009
Untreated	78.57	59.79-90.04	0.002

13.5 Discussion

This study demonstrates that administration of an internal teat sealant containing chlorhexidine at the last milking of the lactation resulted in the lowest prevalence of intramammary infections with any pathogen observed at calving when compared to treatment with a conventional teat sealant or no treatment. The incidence of clinical mastitis during the first 34 days of the dry period was significantly lower in quarters treated with chlorhexidine-containing or commercial teat sealant than in untreated quarters. The incidence of clinical mastitis caused by the challenge organism during this period was not significantly different between groups treated with either teat sealant but was significantly lower in them than in the untreated group. The lower prevalence of intramammary infections after calving in the quarters treated with the chlorhexidine-containing teat sealant should result in a lower prevalence of mastitis throughout lactation, as non-infected cows at calving are less likely to develop mastitis (Barkema *et al.* 1998; Woolford *et al.* 1998). The modest reduction of intramammary infections after calving was due to bacteria different from the challenge organisms. This suggests that the risk of infection by other organisms was reduced. However, as the prevalence of other organisms was not a subject of interest, this *en passant* observation is not a definitive conclusion.

It would be expected that microbial challenge under natural conditions would be less intense than in the present study. The challenge broth contained a concentration of bacteria that would not be expected to occur under natural conditions. Moreover, quarters were exposed to challenge with a single strain of *Strep. uberis*. This differs from the situation in natural conditions, in which animals would be expected to encounter a diverse microbial challenging flora. Despite these caveats, the challenge model used in the present study was valid, since both the model and the strain used for the present study have been used previously with success. This model was shown to be highly effective in causing intramammary infections in untreated quarters in the early dry period (Fernandez 2007; Petrovski *et al.*, 2011, unpublished). Hence, the results are applicable to the external population. Only cows with <200,000 somatic cells/mL and no history of clinical mastitis in the previous lactation were included in the present study. Cows from a single farm were used in the current study to prevent inter-farm variability (Barkema *et al.* 1999; Godden *et al.* 2003; Newton *et al.* 2008). As the effect of the individual cow on the results of challenge experiments is generally significant (Huxley *et al.* 2002; Newton *et al.* 2008), this was included in the modelling. The split study design, in which two quarters per cow were treated with the novel sealant and the other two quarters with a conventional sealant, that was used in the present study may actually underestimate the true efficacy of the prevention capabilities of the test items at the cow level (Berry *et al.* 2003). Regardless, any such effect would affect both products used in the study equally and not bias the results towards any treatments.

The number of cows in the present study was adequate to allow rejection of the null hypothesis (power analysis not shown). Although relatively few cows were enrolled in the negative control group, this approach was taken to limit the unnecessary suffering of animals, given that the challenge procedure is highly effective. The treated and untreated groups had similar infection status before drying off, as only two glands (one in the chlorhexidine-containing teat sealant treated and one in the untreated group) were infected with a major pathogen at drying off (Table 13.6). The study design did not allow for detailed analysis of microbial results and hence, no lengthy discussion on this will be attempted. The overall effect of treatment on the prevalence of intramammary infections was significant. The study did not attempt to estimate the effect of the teat sealants on existing intramammary infections. As chlorhexidine was incorporated in the novel teat sealant solely for its local activity in the teat canal and teat cistern, it was assumed that

cows with pre-existing infection that cured did so as a result of self-cure rather than the effect of the introduced chlorhexidine in the teat sealant. This could be the subject of further research. Additionally, the lengths of the dry periods were also similar (103 and 96 days for the treated and untreated cows, respectively). Hence, differences in the length of the dry period cannot explain the observed differences in quarter intramammary infections at calving. Regardless, a higher probability of infection at calving would be expected in treated quarters due to their longer dry period (Rindsig *et al.* 1978; Berry and Hillerton 2007).

Six quarters that developed clinical mastitis during the first 34 days after treatment and were treated for clinical mastitis according to the study protocol yielded *Strep. uberis* after calving. Previous work by this group has confirmed by Pulse-Field Gel Electrophoresis that all clinical cases during the dry period and most subclinical infections after calving were caused by *Strep. uberis* identical to the challenge strain (Chapter 12). The *in vitro* tests conducted on the challenge strain have shown a high susceptibility to all beta-lactam antibiotics. The reason for the finding of these infections after calving is not clear. They may have resulted from treatment failure or re-infection.

Since their development in the 1970s, internal teat sealants containing bismuth sub-nitrate have been evaluated in Canada, Ireland, New Zealand, the United Kingdom and the USA. Studies have demonstrated that application of various formulations of teat sealants to uninfected mammary glands is at least as effective as a long-acting DCT, if not better, in reducing the rate of new intramammary infections during the dry period (Meaney 1977; Woolford *et al.* 1998; Berry and Hillerton 2002a; Godden *et al.* 2003; Crispie *et al.* 2004a, 2004b; Cook *et al.* 2005). The chlorhexidine-containing teat sealant used in the present study demonstrated superior protection to that of the conventional teat sealant when measured as the prevalence of intramammary infections with all pathogens after calving. The reason for this benefit, compared to the lack of advantage of combining teat sealant and DCT (Woolford *et al.* 1998), is not clear. However, it appears to support the hypothesis of the study; namely that it is a result of the local activity of chlorhexidine destroying organisms in the teat cavity, either those which invaded before the sealant formed a perfect plug or those introduced with the treatment. On the other hand, antimicrobial concentration from the DCT around the time of calving may be lower than the minimal inhibitory concentrations, leading to increased susceptibility to new infections. Moreover, the teat sealants form a persistent barrier that in the case of the

chlorhexidine-containing teat sealant may protect against organisms invading teats that have the sealant plug loosened from the teat cistern wall, such as leaky teats.

Currently, available teat sealants are not ideal for preventing new intramammary infections during the dry period, inasmuch as a failure to protect 2.0 to 42.5% of quarters has been reported (Woolford *et al.* 1998; Huxley *et al.* 2002; Bradley *et al.* 2010). The most likely reason for a failure of prevention could be the risk of contaminating quarters during treatment, since conventional teat sealants lack constituents with antimicrobial properties (Bradley and Green 2004). Aseptic technique during administration is paramount for the infusion of any intramammary product, but it is known that farming practice varies widely, possibly based on the assumption that it is less important when infusing antimicrobial formulations (Woolford *et al.* 1998). Prophylactic administration of DCT to uninfected quarters could predispose to new intramammary infection due to: disruption of the epithelial integrity of the teat canal; accidental introduction of mastitis-causing organisms from around the teat end particularly when they are resistant to the antimicrobial used; or disruption of the normal micro flora (Williamson *et al.* 1995; Huxley *et al.* 2002; Godden *et al.* 2003; Crispie *et al.* 2004b). The impact of new intramammary infections introduced by this procedure is difficult to quantify: they may persist until the next lactation but also could result in acute onset of clinical mastitis before the active involution of the gland is finished (Smith *et al.* 1985; Bradley and Green 2004). The coagulase-positive staphylococci present at calving and absent at drying-off in quarters treated with commercial teat sealant may have been introduced during treatment or have invaded the teat canal in the early dry period from micro-skin lesions surrounding the teat canal. This is supported by the lower prevalence of minor mastitis-causing organisms in quarters treated with chlorhexidine-containing teat sealant when compared to quarters treated with commercial teat sealant and untreated quarters (Table 13.6). Therefore, to overcome this shortcoming of currently-available teat sealants, the addition of an appropriate antimicrobial agent, such as chlorhexidine in the novel internal teat sealant, appears to be worthwhile. Confidence of farmers in sealant may increase with the presence of antimicrobial compounds in the formulations. The sealant provides a barrier throughout the dry period against many microbial species that gain entry into the gland through the teat canal. Furthermore, an effective antimicrobial will inhibit or kill mastitis-causing organisms which evade the teat sealant plug (Ryan *et al.* 1998; Godden *et al.* 2003). This possibly explains the observed lower number of infected

quarters at calving which was found in the present study in quarters treated with the novel internal teat sealant when compared to untreated quarters and those treated with the commercial teat sealant. The results support a view that chlorhexidine-containing teat sealant reduced the ability of major and minor mastitis-causing organisms to penetrate the teat canal and establish intramammary infections during the dry period.

13.6 Conclusion

This study demonstrates positive effects from the use of an existing and a novel internal teat sealant containing chlorhexidine in cows with low somatic cells and no history of clinical mastitis during the previous lactation on the prevalence of new intramammary infections after calving and on the incidence of clinical mastitis in the non-lactating period.

13.7 Acknowledgments

Bomac Laboratories Ltd is thanked for the financial support of this study. The assistance of Duncan Hedderley (*The New Zealand Institute for Plant & Food Research Limited*, Palmerston, North, New Zealand) with the preliminary statistical analyses of this study is greatly appreciated. The staff from the *Agricultural Farm Services*, Massey University are also thanked for their assistance with the animal husbandry. KR Petrovski's position at Massey University is supported by *Bomac a company of Bayer Ltd*.

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MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

**STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS**

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Kiro R Petrovski

Name/Title of Principal Supervisor: Prof Norman B Williamson

Name of Published Paper: Efficacy of a novel internal dry period teat sealant containing 0.5% chlorhexidine against experimental challenge with *Streptococcus uberis* in dairy cattle

In which Chapter is the Published Work: Chapter 13

What percentage of the Published Work was contributed by the candidate: 70%


Candidate's Signature

11 July 2011
Date


Principal Supervisor's signature

11/7/2011
Date

Chapter 14

General Discussion

14. General discussion

This thesis aimed to validate and further develop strategies for mastitis management. The studies provide more insight into antimicrobial susceptibility (Part One), the pharmacokinetics of penicillin G administered by the intramammary route (Part Two), the effects of a novel mastitis treatment of heifers before calving on mastitis, production and reproduction (Part Three) and the efficacy of a novel internal teat sealant containing chlorhexidine against an experimental challenge (Part Four).

Bovine mastitis is regarded as the most costly production disease to the dairy industry worldwide (Petrovski *et al.* 2006; Halasa *et al.* 2007; Huijps *et al.* 2008). The effect of any disease is affected by its prevalence. Therefore, strategies to decrease the prevalence of the disease are required and for mastitis these are developing continuously. As stated previously in the thesis, the prevalence of mastitis on a dairy farm is affected by the number of infected cows and the duration of each intramammary infection. Therefore, to reduce the prevalence of mastitis, strategies that influence both factors are addressed in this thesis. The strategies discussed show potential to reduce the prevalence of intramammary infections on dairy farms, which should be financially advantageous to the farmer and the dairy industry.

Some limitations to the studies included in the thesis were addressed in the discussion of each Chapter. Therefore, studies reported in the thesis vary in their external validity with a limited external validity in Chapter 10 (Treatment before calving of heifers for mastitis improves their reproductive performance, but not their milk production) to others that are completely applicable to the external population *i.e.* Chapter 6 (Correlation of agar disk diffusion and broth microdilution methods when testing the antimicrobial susceptibility of *Staphylococcus aureus* and streptococci isolated from bovine milk samples collected in New Zealand).

There was a dearth of studies addressing the topics covered in this thesis previously in pasture based systems, which highlights the value of the research reported in this thesis.

14.1 Part One

Shortening intramammary infections can be achieved by effective treatment. To increase the success of treatment, the antimicrobial used should be efficacious against the

causative organisms and achieve an effective concentration at the site of infection. The most common way to select an efficacious therapy to treat an infectious disease is to assess the antimicrobial susceptibility of a particular causative organism. Part One of the thesis concentrated on the aetiology of mastitis in New Zealand and the susceptibility of mastitis-causing organisms. It started with a brief overview of current knowledge on antimicrobial resistance (Chapter 2) and continued through four chapters (Chapters 3 to 6) addressing the susceptibility of mastitis-causing organisms isolated from milk samples collected in New Zealand.

Chapter 3 addressed the aetiology of mastitis in New Zealand, based on milk samples submitted to five commercial diagnostic veterinary laboratories located throughout New Zealand. The importance of various mastitis-causing organisms and their seasonal distribution were documented. This information aimed to assist practicing veterinarians to know the most likely causes of bovine mastitis at various stages of lactation. However, the analysis failed to identify a strikingly different distribution or occurrence of any organism that would make seasonal guidance possible. Therefore, the identity of causative organisms should routinely be confirmed by culturing. Hopefully, in the near future, simple and affordable tests will be available for the cow-side identification of the common mastitis-causing organisms. These may aid in making treatment decisions.

An important finding of this study, acknowledging the possible bias of the data toward problem cases, was that isolation of *Staph. aureus* is common throughout lactation as opposed to the commonly held belief that this organism is common in later season (in New Zealand traditionally after Christmas). This finding emphasises the need for better education of farmers on the preventive measures for contagious mastitis that have been available for years (Plastridge 1958; Dodd *et al.* 1969; Philpot 1969). The high prevalence of isolations early in lactation indicated possibility of poor culling or dry cow therapy policies.

The second most commonly isolated mastitis-causing organism was *Strep. uberis*. Other common mastitis-causing organisms in New Zealand were coagulase-negative staphylococci, *Strep. dysgalactiae*, *Bacillus* spp. and coliforms. The finding that *Strep. uberis* is common in New Zealand was not un-expected, as previous reports have identified this pathogen as the most important for the New Zealand dairy industry (McDougall 1998; Douglas *et al.* 2000; McDougall *et al.* 2007). Regional variation in the

distribution of this organism was detected, probably indicating variations in the weather and ground conditions. It is also possible that there are regional differences in management. This is an area that requires further research. It may be inappropriate to take reports from one region as being indicative of a nationwide situation. This should be taken into account when one is preparing updates of the national mastitis management programme.

Chapter 4 reported the susceptibility to antimicrobials of mastitis-causing organisms using the same data-set for mastitis-causing organisms as for Chapter 3. Analysis of the results of antimicrobial susceptibility of bacterial isolates from milk samples from dairy cows in New Zealand provides useful data for surveillance purposes and a baseline for identifying changes in antimicrobial sensitivity in this population. The susceptibility of *Staph. aureus* in New Zealand has risen from the previous surveys in the 1960s (peer-reviewed sources), and 1970s and 1990s (non-peer-reviewed sources), whilst for streptococci it has fallen. Based on this data-set, mastitis treatment products available in New Zealand, with the exception of lincosamides and aminoglycosides, should be efficacious. The use of aminoglycosides is not justified so their withdrawal from the market in 2010 will have little impact on treatment efficacy. Interestingly, there are isolates of *Strep. uberis* with decreased susceptibility to isoxazoly-*penicillin* and susceptible to penicillin, providing an indication of changes in the penicillin-binding proteins. This may have implications for human health, as streptococci are known as easy donors of resistance genes within and between species (Roberts and Brown 1994; Hakenbeck *et al.* 1998; Martel *et al.* 2005). Therefore, this is an area that requires monitoring and further research.

Chapter 5 compared the antimicrobial susceptibility of *Staph. aureus*, *Strep. dysgalactiae* and *Strep. uberis* isolated from milk samples collected in New Zealand or the USA. The proportion of susceptible isolates from New Zealand differed from that of isolates from the USA. For many of the antimicrobial/mastitis-causing organism combinations differences were found in the size of the zones of inhibition for organisms from the two countries. As it was postulated that the diameters of zones of inhibition can be correlated with the MIC values (Walker 2006) this was identified as an area requiring a further research. On this basis, there was an indication that the doses for various antimicrobials used for treatment of mastitis should differ between the two countries. This supported the necessity for periodic local and national surveys of susceptibility patterns and adjustment

of treatment protocols, if indicated. Discordant isolates of *Strep. uberis*, susceptible to penicillin and resistant to isoxazolyl-penicillins, particularly oxacillin were also found in this experiment. Interestingly, there were also discordant isolates susceptible to erythromycin but resistant to lincomycin. Such isolates of streptococci may be of clinical importance, not only for treating bovine mastitis, but also for other pathogens such as human streptococci, due to the predisposition of streptococci to transfer genes leading to the use of macrolides, and particularly lincosamides, for treatment of bovine mastitis to be discouraged. Furthermore, the susceptibility of streptococcal isolates to tetracyclines was low, with the exception of isolates of *Strep. uberis* from New Zealand. Therefore, the use of tetracyclines for mastitis caused by streptococci should be discouraged. This is supported by the previous reports on unfavourable pharmacokinetics of these drugs in the mammary gland (Barza *et al.* 1975; Fang and Pyorala 1996; Jung *et al.* 1997) and decreased activity in milk (Owens and Watts 1987; Fang and Pyorala 1996; Jung *et al.* 1997).

Chapter 6 compared the results obtained using the disk diffusion method, which is routinely employed in veterinary diagnostic laboratories, to the more definitive MICs obtained by the broth microdilution method. Thus, this Chapter addressed one of the knowledge gaps identified in Chapter Five. A high correlation between the inferences drawn from the disk diffusion and microdilution techniques was common, but for some antimicrobial/mastitis-causing organism combinations significant misclassifications were found. Hence, results from veterinary diagnostic laboratories should be interpreted with care, particularly in the face of unexpected clinical outcomes. The disagreement in the result between the two tests was most likely due to incorrect breakpoints. Therefore, new breakpoints to the antimicrobials used to treat common mastitis-causing organisms at a species level are required. It was proposed that when reporting the results of the agar disk diffusion test, laboratories and authors should include the sizes of zones of inhibition. The current interpretive criteria should also be added to allow veterinarians to make more informed decisions on the choice of antimicrobial for treatment of mastitis.

In summary, Part One of this thesis identified some strategies that should be considered in the management of bovine mastitis presented in order of importance:

- Beta-lactams (particularly penicillin) are a preferred treatment for bovine mastitis caused by gram-positive cocci.

- Aminoglycosides, lincosamides and macrolides should be avoided for treatment of mastitis caused by gram-positive cocci.
- Tetracyclines and quinolones should be avoided for treatment of streptococcal mastitis.
- Periodic surveys of aetiology of mastitis and susceptibility patterns of mastitis-causing organisms on national level to follow the changes in the trends of aetiology and susceptibility.
- Antimicrobial susceptibility testing should be conducted using the antimicrobials of interest instead of antimicrobial class representatives.

14.2 Part Two

Part Two of the thesis concentrated on the effects of various treatment regimes and milking frequency on some of the pharmacokinetic properties of penicillin G administered by the intramammary route to healthy lactating dairy cattle. It started with a brief overview of current knowledge on the pharmacokinetics of antimicrobials administered by the intramammary route (Chapter 7) and in Chapter 8 addressed the effects of extended treatment and decreased milking frequency on the elimination time, proportion of recovery of the drug from milk and the time above the MIC. The antimicrobial studied was penicillin G, from the beta-lactam class, which was identified in Part One as the preferred treatment for bovine mastitis in New Zealand. Reduced milking frequency resulted in extension of the elimination time, smaller amounts of penicillin G recovered from milk and an effective concentration throughout the inter-treatment interval and beyond. Extended treatment (six *vs.* three treatments) resulted in a similar elimination time and amounts of penicillin G recovered from milk, but longer periods of effective concentration. Intuitively it may seem that extended treatments should result in prolonged elimination time after the last treatment but this was not the case in this study. Thus, changing the treatment regime requires careful investigation of the pharmacokinetic properties of the drugs administered by the intramammary route in healthy lactating cows before correct recommendations can be made. Interestingly, similar lengths of effective concentrations were achieved with both reduced milking frequency and extended treatments. Therefore, using the pharmacokinetic/pharmacodynamic approach, it was postulated that these two management strategies should result in increased cure rates. However, the reduced milking frequency was tested

only in cows milked once-a-day throughout lactation. The pharmacokinetic parameters in cows milked twice daily and changed to once-a-day milking may be different due to alterations in mammary gland physiology during the adaptation period (Linzell 1971; Knight *et al.* 1994; McManaman and Neville 2003) such as opening of the tight junctions and transient changes in milk composition. This area requires further research. The lower proportion of recovered penicillin G from milk in cows milked once-a-day than from either group milked twice daily may be due to the greater systemic absorption from the udder and/or local degradation of the antimicrobial, as there was no evidence of the antimicrobial residing in the udder for a longer period.

In summary, Part Two of this thesis identified further strategies that should be considered in the management of bovine mastitis in order of importance:

- To extend treatment and decrease milking frequency as measures to improve the cure rates after administration of penicillin G by the intramammary route.
- To investigate elimination times for all new antimicrobial formulations in cows milked at different milking frequencies.

14.3 Part Three

Part Three of the thesis focused on reducing the number of infected animals, in this case heifers. It started with an overview of current knowledge on mastitis in heifers (Chapter 9). Chapter 10 reports an investigation on a single farm of the effects of a novel treatment for mastitis before calving in heifers on the rate of clinical mastitis, milk production and reproductive performance.

Treatment of heifers before calving on this farm resulted in less clinical mastitis and improved reproductive performance, but did not increase milk production. Further larger studies are required to establish the relationship of treatment and milk production in heifers in New Zealand. Treated heifers started the season with higher somatic cell scores. This was likely due to irritation caused by the treatment product. However, it was not possible to compare this finding with available literature as this issue has not been specifically addressed and requires further research. Treatment of heifers before calving improved the reproductive performance in the first lactation of treated heifers compared to controls. This should result in more compact calving in the second lactation and hopefully better life-long productivity.

In summary, Part Three of this thesis evaluated a novel treatment for mastitis in heifers before calving and its effects on the subsequent productivity of treated heifers. This strategy should be considered in herds with a high prevalence of mastitis in heifers in the early lactation. However, further studies are required before this strategy becomes a routine recommendation.

14.4 Part Four

Part Four of the thesis continued the focus on preventing intramammary infections in dairy cows by treating them at drying off. An experimental challenge study design was used to test the efficacy of a novel internal teat sealant containing 0.5% chlorhexidine during the dry period after administration by the intramammary route at drying-off. Prevention of new intramammary infections during the dry period should significantly lower the prevalence of mastitis in a herd. This is particularly important for New Zealand's seasonal dairying where all cows on a farm are dry at once. Thus, fewer infected cows at calving should help keep the prevalence of mastitis low when mastitis management measures are in place.

Part Four of the thesis started with a critical overview of current knowledge on internal teat sealants and their limitations (Chapter 11) and continued through Chapters 12 and 13 that addressed the efficacy of a novel internal teat sealant containing 0.5% chlorhexidine administered by the intramammary route at drying-off against experimental microbial challenge during the dry period.

Chapter 12 found the two test formulations being evaluated for use as internal teat sealant to be at least equivalent in efficacy to the commercial product. This was confirmed for the level of clinical mastitis caused by the microbial challenge during the dry period and for the somatic cell scores in early lactation after calving. The study lacked sufficient power to demonstrate differences in the prevalence of intramammary infections after calving. The study reported in Chapter 12 was designed in such way that all quarters of a cow were in the same treatment group.

Chapter 13 used a design providing within cow positive control of treated cows and untreated controls. All quarters of negative control cows were left un-treated, whilst treated cows had two contra-lateral quarters treated with the teat sealant containing chlorhexidine and the remaining two were treated with conventional teat sealant. The

aim was to estimate the efficacy of the teat sealants to prevent clinical mastitis in the early dry period after experimental challenge and in the late dry period after natural challenge from the environment. Quarters treated with teat sealants were less likely to have an intramammary infection after calving and had a lower incidence of clinical mastitis during the early dry period than untreated controls in this challenge study. The study was designed to show equivalence and not difference in the protective ability of the two sealants. A tendency toward better protection in the late dry period by the teat sealant containing chlorhexidine was characterised by a lower prevalence of intramammary infections after calving. This was in-line with the postulate that the internal teat sealants in the late dry period (before calving) fail to mould to the expanding teat when the teat cavity increases in volume due to the rapid accumulation of colostrum. This forms channels between the sealant and the teat wall allowing entry of mastitis-causing organisms from the environment. A preliminary investigation by x-ray examination of treated teats supported this notion (data not shown). The presence of chlorhexidine in the teat sealant aims to kill the microbes that gain entry in this way; thus, the incidence of intramammary infections during the dry period should be lower than in quarters protected with the conventional product. A study not reported in this thesis, demonstrated that the concentration of chlorhexidine in the sealant at calving was practically the same at calving as at administration. Further research is required to confirm the postulate of local activity of chlorhexidine incorporated in internal teat sealants. Larger field studies are also required to investigate the existence of differences in the protective ability of the chlorhexidine-containing and conventional teat sealant. In view of the demonstrated efficacy of these products, it is unlikely the difference will be demonstrated easily, except by enrolling of thousands of cows per group and this is likely to be cost prohibitive.

In summary, Part Four of this thesis identified a new strategy that should be considered in the prevention of the acquisition of intramammary infections during the dry period.

In conclusion, this thesis identified a number of strategies that should be considered by the National Mastitis Advisory Committee, veterinarians, milk quality advisors and farm advisors when planning up-dates and development of new mastitis management programmes. The importance of these strategies for individual farms will vary dependent on their existing mastitis management. The best way to educate farmers, veterinarians and advisors would be for the pertinent findings from this work to be incorporated into materials produced as a part of the 'Smart SAMM' extension effort.

14.5 Further research needs identified

Each part of the thesis identified some gaps in current knowledge on mastitis in New Zealand and elsewhere. Avenues for future research have been identified as a result of the work contained in this thesis. These are listed below.

- Conducting clinical pharmacokinetic studies in udders with mastitis (clinical and subclinical).
- Periodic national surveys of the farm-to-farm variability in the aetiology of bovine mastitis in New Zealand.
- Effects of various levels of prevalence of intramammary infections in a herd on the rate of new intramammary infection in heifers.
- Determining reasons for between-island variability in the susceptibility of mastitis-causing organisms.
- Establishing new susceptibility breakpoints to the antimicrobials used for mastitis treatment for common mastitis-causing organisms at a species level.
- Investigate reasons for the trend of increasing susceptibility of *Staph. aureus* isolates from bovine milk samples collected in New Zealand to penicillin over time, such as by investigating the prevalence of beta lactamase producing strains.
- Determining reasons for the trend of decreasing susceptibility of *Strep. uberis* isolates from bovine milk samples collected in New Zealand to isoxazolylic penicillins in recent times such as investigating changes in the penicillin binding proteins.
- Investigating the ability of *Strep. uberis* to donate resistance genes to other streptococci and other bacteria.
- Investigating pharmacokinetic parameters due to changes in mammary gland physiology during the adaptation period in cows milked regularly twice daily then switched to once-a-day milking.
- Defining the fate of antimicrobials administered by the intramammary route and residing for long periods in the udder (*i.e.* in cows milked once-a-day).
- Further investigation into the relationship of treatment of heifers before calving and milk production.
- Determination of the life-long productivity and longevity of treated heifers.

- Examining the effects of various products used for treatment of mastitis of heifers on the somatic cell count after calving.
- Investigating in the field, the differences in protection against new infections in the late dry period offered by chlorhexidine-containing and conventional teat sealants.
- Further investigation of the association of the weather and ground conditions, coupled with various farm and mastitis management practices to the epidemiology of *Strep. uberis* mastitis.

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