Phylogenetic and antibiotic resistance variance amongst mastitis causing \textit{E. coli}: The key to effective control.

By

Daniël Johannes Goosen

13211447

Submitted in partial fulfilment of the requirements for the degree

MAGISTER OF ENVIRONMENTAL SCIENCE

School of Environmental Sciences and Development
North-West University: Potchefstroom Campus
Potchefstroom, South Africa

Supervisor: Prof. C.C. Bezuidenhout

April 2012
ABSTRACT

Environmental pathogens, such as *Escherichia coli* and *Streptococcus uberis*, are currently the major cause of mastitis within dairy herds. This leads to severe financial losses, lower production rates and deterioration of the general health of the herd. *E. coli* mastitis is becoming a major threat to high milk-producing dairy herds. This is because of its increasing resistance to antibiotics, rendering antibiotic treatment regimes against *E. coli* infections mostly ineffective. The aim of this study was to develop a method to select mastitis causing *E. coli* isolates for the formulation of effective herd specific vaccines. Two methods, namely a genotyping method (Random Amplification of Polymorphic DNA; RAPD) and an antibiogram based method, were used. A dairy farm milking approximately 1000 Holstein cows in the Darling area, Western Cape Province, was selected for this study. The study was conducted over a period of 48 months and mastitis samples were analysed for mastitis pathogens. Antibiogram testing (disk diffusion method) and an in-house developed RAPD analysis method were used to analyse the *E. coli* isolates. A total of 921 milk samples were analysed from which 181 *E. coli* isolates were recovered. The number of all other common mastitis pathogens combined was 99 isolates (*Streptococcus uberis* 18, *Streptococcus dysgalactiae* 46, *Streptococcus agalactiae* 1, *Staphylococcus epidermidis* 21, *Arcanobacterium pyogenes* 13). All *E. coli* isolates, except for one, were resistant to at least three antibiotics. Antibiotic variance profiles were also highly erratic. The RAPD analysis revealed high levels of polymorphisms and clear epidemiological trends were observed over time. No similarities in the variance profiles between the antibiotic variance data and phylogenetic data were observed. Formalin inactivated autogenous vaccines were produced containing *E. coli* isolated from the herd. The vaccines were formulated using the RAPD or antibiogram data of the *E. coli* isolates. A total of 5 vaccines were formulated using RAPD data (R-vaccines) and one vaccine was formulated using antibiotic variance data (A-vaccine). The RAPD formulated vaccines were more effective than the antibiotic variance formulated vaccine. After each R-vaccination, the number of *E. coli* mastitis cases declined within the herd. The A-vaccinations seemed to have had no effect, which lead to a rise in *E. coli* mastitis cases. RAPD analysis on new emerging isolates was able to detect genetic variation from vaccine strains, which in turn facilitated the formulation of new updated vaccines with higher effectiveness than the previous vaccine. Mastitis data prior to and after the vaccination period revealed significant higher incidences of mastitis in the herd than during the vaccination period. This study demonstrated that sufficient
sampling practices coupled with a reliable genotyping method, resulted in the formulation of updatable vaccines which were highly effective in controlling *E. coli* mastitis within the herd.

**Keywords:** *E. coli*, Mastitis, RAPD, Antibiotic resistance, Autogenous vaccines
ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following persons and institutions for their contributions and support towards the completion of this study:

Prof. C.C. Bezuidenhout, for allowing me to present this research project for the M.Env.Sci and the patience and guidance he has given me;

Robert and Chris Strake, for allowing me to use their dairy herd as the study group and the on farm monitoring data that they provided;

Disease Control Africa, for my employment, for assisting in producing the vaccines and allowing me to present my work for this study;

IDEXX Laboratories, Dr. Maryke Henton, for the bacterial sample analysis and performing most of the antibiogram tests;

My wife, Vanessa, for the unlimited support and love she has given me and her assistance in formatting the dissertation;

My father, for the wisdom and support he has provided.
DECLARATION

I declare that the dissertation for the degree of Master of Environmental Science at the North-West University: Potchefstroom Campus hereby submitted, has not been submitted by me for this degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

........................................... ...........................................
Daniël Johannes Goosen Date
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td><strong>CHAPTER 1: INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 General introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Problem statement</td>
<td>2</td>
</tr>
<tr>
<td>1.3 Research aim and objectives</td>
<td>3</td>
</tr>
<tr>
<td><strong>CHAPTER 2: LITERATURE REVIEW</strong></td>
<td>4</td>
</tr>
<tr>
<td>2.1 Bovine mastitis</td>
<td>4</td>
</tr>
<tr>
<td>2.1.1 Overview</td>
<td>4</td>
</tr>
<tr>
<td>2.1.2 Impact of mastitis on dairy herds</td>
<td>5</td>
</tr>
<tr>
<td>2.2 <em>Escherichia coli</em></td>
<td>6</td>
</tr>
<tr>
<td>2.2.1 Species characteristics</td>
<td>6</td>
</tr>
<tr>
<td>2.2.2 Antibiotic resistance</td>
<td>7</td>
</tr>
<tr>
<td>2.2.3 Diseases caused by <em>E. coli</em></td>
<td>9</td>
</tr>
<tr>
<td>2.3 <em>E. coli</em> bovine mastitis</td>
<td>11</td>
</tr>
<tr>
<td>2.4 <em>E. coli</em> mastitis vaccines</td>
<td>14</td>
</tr>
<tr>
<td>2.5 Methods for isolation, identification, antibiotic resistance</td>
<td>16</td>
</tr>
<tr>
<td>determination, serotyping and genetic fingerprinting</td>
<td></td>
</tr>
<tr>
<td>2.5.1 Sampling, isolation and identification methods</td>
<td>16</td>
</tr>
<tr>
<td>2.5.2 Antibiotic resistance determination</td>
<td>17</td>
</tr>
<tr>
<td>2.5.3 Serological typing methods</td>
<td>18</td>
</tr>
<tr>
<td>2.5.4 Genetic fingerprinting methods</td>
<td>20</td>
</tr>
<tr>
<td>2.5.4.1 Random Amplified Polymorphic DNA</td>
<td>20</td>
</tr>
<tr>
<td>2.5.4.2 Other molecular markers</td>
<td>21</td>
</tr>
<tr>
<td>2.5.4.3 Phylogenetic tree construction</td>
<td>24</td>
</tr>
<tr>
<td>2.6 Autogenous vaccines</td>
<td>24</td>
</tr>
<tr>
<td>2.7 Summary of literature</td>
<td>26</td>
</tr>
</tbody>
</table>
(iv) Autogenous vaccine formulation, application and the monitoring of
E. coli isolates within the vaccination period............................66

6.2 Recommendations ..................................................................................67

REFERENCES ..............................................................................................69
APPENDIX A ..............................................................................................90
APPENDIX B ..............................................................................................95
APPENDIX C ..............................................................................................97
LIST OF FIGURES

Figure 4.1: A graph illustrating the monthly numbers of lactating cows bearing clinical mastitis as well as the number of cows that died or were slaughtered each month.................35

Figure 4.2: A graph illustrating the monthly number of mastitis causing pathogens isolated during the sampling period..................................................36

Figure 4.3: The total mastitigen composition of all the pathogens that were isolated throughout the sampling period.........................................................37

Figure 4.4: The total number of E. coli isolates isolated each month and the trend line indicating the average number of isolates over every 2 months. The vaccines administered each month are also indicated below the months.................................................................38

Figure 4.5: The monthly numbers of the total mastitigen isolates are depicted as percentages of the number of milk samples taken each month.........................................................39

Figure 4.6: The susceptibility patterns of all the E. coli isolates combined to each antibiotic as well as the averages of all antibiotics combined .................................................................40

Figure 4.7: Examples of ethidium bromide stained 1.8% (w/v) agarose gels illustrating the banding patterns generated by the RAPD reactions. The first and the last lanes of each gel contain the molecular markers and the other lanes contain the RAPD reactions The E. coli isolate numbers of each lane are presented in Appendix C. D) Lanes 3 and 4 is an example of a reproducibility test .........................................................44
Figure 4.8: The phylogenetic tree constructed from RAPD data of the first 10 isolates

Figure 4.9: The phylogenetic tree of the 100 selected *E. coli* isolates constructed by using the RAPD analysis data. The vaccine isolates are also indicated.

Figure 4.10: The antibiotic variance tree of the 100 selected *E. coli* isolates constructed by using the antibiogram data. The vaccine isolates are also indicated.
LIST OF TABLES

Table 4.1: The sequences of the four primers that were selected to undergo RAPD reaction development...........................................................................................................41

Table 4.2: The numeric analysis of the RAPD raw data extracted from the agarose gels ............................................................................................................................43

Table 4.3: The isolate numbers included in the phylogenetic and antibiotic resistance variance trees of *E. coli* pure cultures isolated during the sampling period...........................................................................................................46

Table 4.4: Types of autogenous vaccines produced with their *E. coli* isolate composition and the months the vaccines were administered to the dairy herd ................................................................................................................52
CHAPTER 1
INTRODUCTION

1.1 GENERAL INTRODUCTION

One of the greatest problems faced by the dairy industry is bovine mastitis. Mastitis leads to severe financial losses to dairy farmers due to a reduction in milk production, lower milk quality and in severe cases death of cows (Sørensen et al., 2010). In the past, the contagious pathogens were the main cause of mastitis within dairy herds. These pathogens occur mainly within the cow’s udder and are transmitted by horizontal transfer. However, over the past few years the contagious pathogens became less prominent and the environmental pathogens are becoming the main causes of mastitis. The latter occur naturally in the cow’s surroundings. Escherichia coli have been identified as one of the most prominent pathogens involved in environmental mastitis (Dufour et al., 2009).

\textit{E. coli} infections are common in high milk-producing dairy cows and causes infection and inflammation of the mammary gland mainly around calving and during early lactation. This leads to striking local and sometimes severe systemic clinical symptoms. These clinical symptoms may significantly affect the productivity of the affected cows in dairy herds. Severe cases of \textit{E. coli} mastitis may also lead to several deaths per year within the herd (Hazlett et al., 1984; Wilson et al., 2009).

The \textit{E. coli} pathogens are mainly known to cause acute clinical mastitis which subsides after a period of time (Burvenich et al., 2003; Günther et al., 2011). However, a study on \textit{E. coli} induced mastitis has revealed the emergence of strains capable of causing chronic mastitis. These strains are able to adhere to and internalize into the bovine mammary epithelial cells more successfully than strains isolated from acute mastitis (Almeida et al., 2011).

Infection in the mammary glands by \textit{E. coli} can result in the release of toxins, which mainly occurs upon death of the bacterial cells. These toxins are one of the main contributors of clinical signs of mastitis and therefore the use of antibiotics for the
treatment of *E. coli* mastitis is not recommended (Petersson-Wolfe and Currin, 2011). Shiga-toxin producing *E. coli* strains have also been detected in mastitis cases, which in turn pose a health risk to humans handling and consuming fresh milk (Kossowska and Malinowski, 2007).

### 1.2 PROBLEM STATEMENT

*E. coli* is very difficult to control and eliminate from dairy herds because of the high genetic variability within the species and the vast number of environmental sources it can be contracted from (Lohuis *et al.*, 1989; Petersson-Wolfe and Currin, 2011). Use of antibiotics is becoming less effective due to high antibiotic resistance transfer rates between bacteria. More *E. coli* strains are becoming resistant to the commonly used antibiotics (Santos *et al.*, 2010). This gives a strong indication that antibiotic resistance monitoring of mastitis causing *E. coli* can be an important tool in controlling mastitis within dairy herds. The disk diffusion method is the most widely used and standardised method for antibiotic resistance determination (Kahlmeter *et al.*, 2009). In addition, hygienic practices can be implemented in herd management which reduce the occurrence *E. coli* mastitis. However, the effectiveness of such practices is still highly variable and unpredictable (Petersson-Wolfe and Currin, 2011).

Preventative control strategies are the most effective means to control mastitis development within dairy herds (Schroeder, 1997). The vast range of *E. coli* strains that can cause mastitis, genetic variation amongst these strains and the lack of correlation between virulence factors and the severity of mastitis make mastitis vaccine formulation challenging (Silva *et al.*, 2009; Suojala *et al.*, 2011). These factors have rendered many *E. coli* mastitis vaccines ineffective (Denis *et al.*, 2009; Silva *et al.*, 2009). Furthermore, only a limited number of antigens can be included in a specific vaccine and vaccine strains must be homologous to present field strains (Funk *et al.*, 2009; Cooper, 2010 a).

The above mentioned factors accentuate the need to employ analysis methods to aid in the selection of *E. coli* strains for mastitis vaccine formulation. Genomic based methods would be the preferred methods since most of the inherent properties of bacteria are located on the genome (Gomes *et al.*, 2005). The random amplified polymorphic DNA (RAPD) method is a PCR based technique which is fast and
relatively inexpensive to perform (Dautle et al., 2002). A previous study has found a strong relation between RAPD generated variation and antigenic variation of different E. coli strains (Russo et al., 2007). Antibiotic resistance variance determination could also be used to select strains for vaccine formulation in order to reduce the occurrence of highly resistant strains. However, the effectiveness of this vaccine strain selection method could be unpredictable. This is due to the fact that antibiotic resistance properties of bacteria mainly occur on less stable genetic elements such as plasmids and transposons (Berge et al., 2005).

There is a great need for more effective measures to control E. coli bovine mastitis in dairy herds. Current control measures are variable in their effectivity and could also be expensive to sustain.

1.3 RESEARCH AIM AND OBJECTIVES

The aim of this study was to determine the genetic variability and antibiotic resistance profiles amongst mastitis causing E. coli isolates and the application of these methods for the formulation of effective farm specific autogenous vaccines.

The objectives were:

i. to develop a fast, reliable and reproducible genotyping method for E. coli.
ii. to determine the antibiotic resistance profiles of E. coli isolates.
iii. to determine whether there is a relation between the antibiotic resistance and phylogenetic profiles of the E. coli isolates.
iv. to formulate and apply autogenous vaccines to a selected dairy herd.
v. to monitor E. coli mastitis incidences and the phylogenetic profiles of the isolates within the vaccinated herd.
CHAPTER 2
LITERATURE REVIEW

2.1 Bovine mastitis

2.1.1 Overview

Mastitis is one of the greatest problems faced by the dairy industry (Sørensen et al., 2010). It not only leads to severe financial losses to dairy farmers, but also poses a significant threat to the welfare of dairy cattle (Green, 2002; Sørensen et al., 2010). Mastitis is defined as the inflammation of the mammary gland caused by microorganisms (mainly bacteria) that invade the udder, multiply and produce toxins that are harmful to the mammary gland (Schroeder, 1997; Lavon et al., 2011).

Bovine mastitis is a very complex disease and is generally categorised into two main forms, namely subclinical and clinical mastitis, both having significant impacts on dairy herds (Lavon et al., 2011). In subclinical mastitis, cows have mastitis but with the absence of any visible symptoms of the disease (Oliveira et al., 2011). There is no gross inflammation of the udder or superficial changes in milk quality and the general health status of the cow appears to be normal. The indicators for subclinical mastitis are a decrease in milk production and a decrease in the nutritional and compositional quality of the milk (Hurley, 2010). Clinical mastitis is defined by the presence of visible symptoms which include swollen quarters, abnormal secretions from the teat and clots or flakes in the milk (Lehtolainen, 2004). The general health condition of the cow may also be mildly to severely affected, depending on the degree of the disease. Loss of appetite, dehydration, rapid pulse, high fever and death may occur (Hurley, 2010).

Three major interdependent factors have been identified that plays a role in the complexity and severity of mastitis, namely: i) the microorganisms as the causative agent, ii) the cow as the host, iii) and the environment in which the cow and microorganisms occur. Over 100 different microorganisms are able to cause mastitis, which vary greatly in their infection routes and the nature of the disease they cause. Cows can contract udder infections at different ages and stages of the lactation cycle,
and they vary in their resistance and defence mechanisms to these infections. The environment influences both the amount and types of bacteria to which the cows are exposed and the resistance of the cows to these bacteria (Schroeder, 1997).

The most common mastitis pathogens occur either in the cow’s udder, known as contagious pathogens, or in the cow’s surroundings, known as environmental pathogens (Jones and Bailey, 1998). The main contagious pathogens are *Streptococcus dysgalactiae*, *S. agalactiae* and *Staphylococcus aureus*. The environmental pathogens are mainly *Streptococcus uberis* and *Escherichia coli*. In the United States of America it was found that the contagious pathogens were the dominant cause of mastitis, but over the past decade the environmental pathogens became more problematic with the importance of the contagious pathogens decreasing. This shift from contagious to environmental pathogen occurrence became apparent when, after the successful control of contagious pathogens was achieved, dairy farms still had problems with increased clinical mastitis cases (Jones and Swisher, 2009). A study in South Africa, however, has found that both contagious and environmental pathogens are still prominent in its dairy herds (Petzer et al, 2009).

### 2.1.2 Impact of mastitis on dairy herds

Mastitis severely impacts the general welfare of dairy herds, which in turn have substantial economic impacts on dairy farmers (Notebaert et al., 2008). Subclinical mastitis affects only milk yield and quality within a herd (Guo et al., 2010). Clinical mastitis, in addition, affects the general health condition of a cow and could lead to death (Pieterse, 2008). However, subclinical mastitis is 15 to 40 times more prevalent than clinical mastitis (Hurley, 2010). Subclinical mastitis is more prone to become a chronic problem within herds leading to sporadic clinical mastitis occurrences (Almeida et al., 2011).

Clinical mastitis indirectly affects the reproductive performance of cows. It was found that mastitis alters the interestus intervals and shortens the luteal phase. Cows that contracted clinical mastitis before the first postpartum artificial insemination have had an increased number of days not pregnant (DNP) as compared to cows without mastitis. A decrease in the general health condition of cows with clinical mastitis also leads to greater culling rates within dairy herds (Ahmadzadeh et al., 2009).
Clinical and subclinical mastitis both severely affect milk yield and milk quality. Milk production of cows bearing mastitis is significantly lower than that of healthy cows. Furthermore, the nutritional quality is lower and the somatic cells count (SCC) is substantially higher (Schukken et al., 2009). The SCC of milk is regarded as the industry’s standard indicator for the general quality of produced milk. It is determined as the total count of white blood cells per millilitre of milk. Normal milk is believed to have a SCC of approximately 200,000 cells/ml or less. An infection in the mammary gland of the udder causes a large influx of somatic cells, predominantly polymorphonuclear neutrophils, which can increase the SCC of milk up to 1 million cells/ml (Guo et al., 2010; Madouasse et al., 2010). Subclinical mastitis commonly contributes a more substantial part to high SCC’s within a herd and is usually a reliable indication of the development of clinical mastitis. (Olde Riekerink et al., 2007; Van den Borne et al., 2011).

All the above mentioned symptoms and effects of mastitis within a herd have considerable financial implications for the dairy farmer. Firstly, clinical mastitis leads to high treatment costs and replacement costs in the case of deceased animals. However, these consequences are overshadowed by financial losses caused by the reduction in milk yield and milk quality (Sørensen et al., 2010). Increased culling rates, increased DNP and induced health stress on mastitis bearing cows reduce milk production. High SCC’s decreases the quality of the milk, in which case the milk processing companies induce significant financial penalties on farmers due to decreased usability of the milk. In most cases, severely affected milk is discarded by the farmer (Madouasse et al., 2010).

2.2 *Escherichia coli*

2.2.1 Species characteristics

*Escherichia coli* is a Gram-negative, non-spore-forming rod that belongs to the family *Enterobacteriaceae*. This organism is facultative anaerobic and occurs naturally in soil and the lower intestine of mammals. Capsules or microcapsules occur in many strains and some strains are motile (Holt et al., 2000).
*E. coli* is traditionally typed into serological strains based on the presence and type of specific antigens. Complete serotyping includes somatic (O), capsular (K) and flagellar (H) antigens. The presence of virulence factors is also used to identify and epidemiologically characterise pathogenic strains (Ballmer et al., 2007; Kausar et al., 2009). Typical strains of *E. coli* are biochemically not difficult to differentiate from other genera, however, it is very difficult to differentiate metabolically inactive *E. coli* strains from *Shigella sp.* (Holt et al., 2000).

*Shigella sp.* and certain *E. coli* strains are genetically highly similar and can cause similar diseases in mammals (Brenner et al., 2005), which also makes genetic differentiation between them difficult. There are however a few biochemical traits, such as the ability to metabolize mucate or acetate, which can distinguish between the two organisms. Furthermore, the lactose permease gene (*lacY*) is commonly used as a genetic marker for distinction. *E. coli* is *lacY* positive and *Shigella sp.* is negative. Current PCR based methods targeting the *lacY* and *uidA* (*β*-glucuronidase) genes are very fast and efficient in discriminating between these two species (Pavlovic et al., 2011).

### 2.2.2 Antibiotic resistance

The extensive use of antibiotics for the control of bacteria in humans and veterinary medicine leads to the selection of resistant bacteria (Santos et al., 2010). These resistant strains are then able to transfer their resistance to other sensitive bacteria. Antibiotic resistance genes mainly occur on plasmids. These plasmids facilitate the easy transfer of resistance between bacteria (Nijsten et al., 1996). Antibiotic usage not only selects for resistance in pathogenic bacteria, but the endogenous flora of the treated individual can also become resistant to antibiotics. The endogenous flora can in turn transfer the resistance genes to new infecting pathogens. Crowding and poor sanitation practices within farm animals are two other factors promoting antibiotic resistance transfer (Van den Bogaard et al., 2001).

*E. coli*, like many Gram-negative bacteria, is intrinsically resistant to hydrophobic antibiotics such as novobiocins, macrolides, rifamycins, fusidic acid and actinomycin D (Brenner et al., 2005). The low permeability of the outer membrane bilayer to lipophilic solutes and active efflux mechanisms of *E. coli* accounts mainly for its
resistance to the above mentioned compounds (Ofek et al., 1994). Resistance to aminoglycosides, beta-lactams, chloramphenicol, sulfonamides, tetracycline and trimethoprim have been acquired by *E. coli* strains from other microorganisms (Alekshun and Levy, 1997; Brenner et al., 2005).

Acquired resistance can develop by means of four distinct mechanisms: i) alteration of the target site, ii) enzymatic detoxification of the antibiotic, iii) decreased drug accumulation and iv) bypassing of an antibiotic sensitive step (Brenner et al., 2005). The first three mechanisms can be mediated by either the acquisition of plasmids carrying resistant genes or by chromosomal mutations (Criswell, 2004; Vignaroli et al., 2011). The fourth mechanism is mainly mediated by horizontal transfer of antibiotic resistance genes on plasmids or transposons (Andam et al., 2011).

Several studies have revealed that antibiotic resistance amongst *E. coli* strains is becoming an increasing problem in the medical and veterinary fields, especially in developing countries (Okeke et al., 2000; Ateba et al., 2008; Ateba and Bezuidenhout, 2008; Santos et al., 2010). Endogenous *E. coli* strains from healthy individuals can acquire antibiotic resistance, which in turn can be transferred to pathogenic strains after infection has occurred (Courvalin et al., 1977). A recent study in the USA found that from 135 mastitis causing *E. coli* isolates, which were tested for resistance against most commonly used antibiotics in veterinary and human medicine, all of them were resistant to two or more antimicrobials (in different combinations). They also carried multiple resistant genes (Srinivasan et al., 2007).

In the North West Province, South Africa, a study has determined the antibiotic resistance patterns of *E. coli* O157 that was isolated from humans, pigs and cattle (Ateba and Bezuidenhout, 2008). Out of the 76 isolates that were recovered a large proportion (52.6% to 92.1%) was resistant to tetracycline, sulphamethoxazole and erythromycin. This occurrence of multidrug resistant *E. coli* O157 accentuates the need for proper hygiene practices on farms, as these pathogens can easily spread to other animals and humans (Ateba and Bezuidenhout, 2008).

A Finnish study has found that mastitis causing *E. coli* isolates were more resistant to antibiotic agents that have been continuously used for several years in dairy cattle (Suojala et al., 2011). The resistance patterns of the *E. coli* isolates are an indication
of which antibiotics have been used for prolonged periods within herds or geographical areas. Routine antibiotic treatment for *E. coli* mastitis is not recommended in Finland in order to reduce the selection pressure of antibiotic resistance amongst the *E. coli* populations (Suojala *et al.*, 2011).

A Chinese study has investigated the use of egg yolk immunoglobulin (IgY), which is produced in chickens using a specific *E. coli* strain, for treating *E. coli* mastitis (Zhen *et al.*, 2008). This method can be used as a therapeutic treatment in diseased animals and has shown promise as an effective alternative to antibiotics. The drawbacks of the method though are the lengthy and tedious production methodology that has not been widely tested on a variety of *E. coli* isolates (Zhen *et al.*, 2008). Genetic variability between isolates could also decrease effectiveness, as in the case with *E. coli* mastitis vaccines (Gregersen *et al.*, 2010).

Another alternative to antibiotic treatment is homeopathy. Various homeopathic compounds are available for different types of mastitis symptoms (Duval, 1997). However, monitoring and correct identification of the different symptoms together with many applications of the remedies required can become tedious for farmers. Due to limited published data available and low effectiveness of homeopathic treatment recorded in previous studies, the outcome of homeopathy for mastitis treatment can be unpredictable (Duval, 1997; Hektoen *et al.*, 2004; Werner *et al.*, 2010).

### 2.2.3 Diseases caused by *E. coli*

*E. coli* strains are genetically diverse and range from non-pathogenic to highly pathogenic strains causing a variety of diseases in mammals. Many strains are considered to be opportunistic pathogens causing only disease when occurring in mammals outside of its natural habitat (the lower intestine). Non-pathogenic strains may also become pathogenic by the acquisition of genes that encode virulence factors, most of which are encoded on extrachromosomal genetic elements (Coetzer and Tustin, 2004). The highly pathogenic strains produce specific toxins and other virulence factors which could lead to severe disease symptoms and death of the host (You *et al.*, 2011).
Although *E. coli* is a natural harmless inhabitant of the lower intestine, pathogenic strains do occur that cause intestinal diseases in mammals, with the most common disease being diarrhoea. These diarrheagenic strains are currently grouped into the following six categories: Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC), and Shiga toxin-producing *E. coli* (STEC) which is also known as Verocytotoxin-producing *E. coli* (VTEC) (Coetzer and Tustin, 2004; Dutta *et al.*, 2011).

*E. coli* strains belonging to these diarrheagenic groups produce a variety of toxins and virulence factors, each which characterise strains into their specific groups (Sekse *et al.*, 2011). The EPEC group causes disease by adhering to the intestinal mucosa to produce the characteristic “attaching and effacing” lesion in the brush border microvillous membrane. Intimin is the main virulence factor and is coded for by the *eae* gene (Trabulsi *et al.*, 2002). On the other hand, the ETEC group mainly causes disease by the production of heat-labile and heat-stable enterotoxins and do not invade the epithelial cells. This group also possesses colonization factors which aid in their pathogenesis (Brenner *et al.*, 2005). Furthermore, EIEC strains are very similar to *Shigella* and they are capable of invading and multiplying in the intestinal epithelial cells (Coetzer and Tustin, 2004). These strains possess invasiveness plasmids and chromosomal genes which are necessary for their virulence (Ephros *et al.*, 1996).

The EAggEC group is characterised by distinct aggregative adherence to HEP-2 cells *in vitro*. Certain types of EAggEC cause diarrhoea and other enteric symptoms such as borborygmi and cramps (Brenner *et al.*, 2005). The main virulence determinants are the presence of the pAA plasmid and the production of the EAST1 toxin (Piva *et al.*, 2003). Diffusely adherent *E. coli* are defined by the presence of the diffuse adherence pattern of the strains to HEP-2 cells. Two surface fimbrial adhesions designated F1845 and AIDA-I are the main virulence factors characterising this group (Coetzer and Tustin, 2004; Brenner *et al.*, 2005).

STEC is responsible for causing haemorrhagic diarrhoea. The main characteristic of this strain of *E. coli* is the production of one or both of the shiga-toxins Stx1 and Stx2 (Botteldoorn *et al.*, 2003). *E. coli* O157:H7 is the prototype organism of this group (Brenner *et al.*, 2005). A study in the North West Province of South Africa has found a
higher prevalence of \( E. coli \) O157 in pigs than in humans and cattle. This indicates that pig farms could be a source for spreading this pathogen within the environment (Ateba et al., 2008). The isolates were identified as \( E. coli \) O157 with the \( E. coli \) O157 rapid slide agglutination test (API 20E). Interestingly, it was found that none of these isolates that were screened by PCR (76 isolates) possessed the shiga toxin genes (Ateba and Bezuidenhout, 2008).

\( E. coli \) is also known for causing several extra intestinal diseases which occur at any site outside the intestinal tract of the host. Urinary tract infections, septicaemia, oedema disease, mastitis, abortion and neonatal meningitis are common diseases caused by various strains of \( E. coli \) (Coetzer and Tustin, 2004). The O groups O7, O18ac, O1 and O6 of \( E. coli \) are frequently associated with neonatal meningitis (Brenner et al., 2005). Keratoconjunctivitis in guinea pig eyes caused by \( E. coli \) have also been reported (Ephros et al., 1996).

### 2.3 \( E. coli \) bovine mastitis

\( E. coli \) mastitis is also known as environmental mastitis. \( E. coli \) is an opportunistic causative agent of bovine mastitis (Passey et al., 2008; Buitenhuis et al., 2011). It causes infection and inflammation of the mammary gland of dairy cows mainly around calving and early lactation. This leads to striking local and sometimes severe systemic clinical symptoms. \( E. coli \) invades the udder through the teat canal where it grows and initiates a prompt inflammatory reaction (Burvenich et al., 2003; Lehtolainen, 2004).

Mastitis caused by the environmental pathogens is traditionally considered to occur sporadically without long lasting effects within the host. The contagious pathogens, on the contrary, are able to persist within the host for prolonged periods of time causing continuous occurrence of mastitis and spreading between quarters and cows (Passey et al., 2008). More recently DNA fingerprinting data suggested that some \( E. coli \) strains have adapted to survive within the udder and cause recurrent mastitis. There is, however, no apparent single factor which is responsible for the persistence of \( E. coli \) infections in the udder (Almeida et al., 2011).

\( E. coli \) mastitis differs from other \( E. coli \) infections because the clinical signs are primarily caused by the host response and not by the pathological changes caused by
bacterial toxins or other damaging factors (Buitenhuis et al., 2011). Previous studies strongly suggested that *E. coli* mastitis is not caused by a limited number of strains with specific characteristics, but that it's caused by a great variety of genotypically different strains that occur in the cow's environment (Ghanbarpour and Oswald, 2010; Suojala et al., 2011). *E. coli* isolates from clinical mastitis possess a variety of uncommon virulence factors which seems to show no correlation to the severity of the disease (Lehtolainen, 2004).

Adhesion and invasion of bacteria to the host epithelia is normally the first step of pathogenesis. This is mediated by a F17 fimbrial adhesion located on the bacterium surface. However, adhesion or attachment of *E. coli* to the mammary epithelium does not play an important role in *E. coli* mastitis. Only a very small portion of clinical mastitis *E. coli* isolates carry the genes encoding F17 related fimbriae (Wilson et al., 1997; Wenz et al., 2006).

The production of cytotoxic necrotizing factor (CNF) toxins is a common virulence factor of *E. coli* (Ghanbarpour and Oswald, 2010). There are two types of these toxins that are produced, CNF1 and CNF2 (Burns et al., 1996). CNF1 production occurs mainly in humans and CNF2 production is common in bovine *E. coli* strains. Not all *E. coli* isolates from mastitis produce CNF2 and no correlation between CNF2 production and disease severity has been found (Lehtolainen, 2004; Wenz et al., 2006).

In the past, serum resistance of *E. coli* causing mastitis has been considered to be an obligatory virulence factor (Lehtolainen, 2004; Blowey and Edmondson, 2010). Further studies indicated that serum sensitive isolates are more common than previously suggested, but the severity of the disease caused by serum resistant strains is higher than with serum sensitive strains. However, there is some indication that serum resistance is not a constant virulence factor in *E. coli* mastitis cases (Lehtolainen, 2004). In general, serum resistance is still the most prevalent virulence factor in *E. coli* mastitis (Wenz et al., 2006). The outer membrane protein TraT is a common virulence factor in *E. coli* isolates from healthy and diseased animals and occurs most commonly in mastitis causing *E. coli* strains. TraT is believed to play an important role in serum resistance of *E. coli* (Açık et al., 2004; Lehtolainen, 2004).
As indicated previously, most studies regarding mastitis causing *E. coli* pathogenesis revealed that there is no association between strain serotype, genotype or the presence of virulence factors and the severity of clinical mastitis. A Finnish study has indicated that certain virulence genes were more prevalent in *E. coli* isolates that were re-isolated after treatment. However, no strain correlation could be confirmed with pulse field gel electrophoresis (Suojala *et al.*, 2011). No mastitis causing subset could be identified with traditional O antigen serotyping due to the fact that bovine mastitis causing *E. coli* do not have a specific antigen O serogroup. In addition, no significant biochemical differences have been found between mastitis causing and faecal *E. coli* (Blum *et al.*, 2008). The only constant characteristic found in mastitis causing *E. coli* is the high degree of genotypic variability between isolates (Ghanbarpour and Oswald, 2010).

Several studies have indicated that the host’s immune response plays a major role in the susceptibility of the cow to *E. coli* mastitis (Lehtolainen, 2004; Sørensen *et al.*, 2009; Suojala *et al.*, 2010). The severity of *E. coli* mastitis is mainly determined by cow factors rather than the pathogenicity of the bacterium itself (Buitenhuis *et al.*, 2011). The ability of neutrophils to sequester and kill the pathogens is a critical step in the host’s defence reaction against *E. coli*. Immune mechanisms in the mammary gland also greatly reduce systemic mastitis signs such as fever, discomfort and temporary reduction in milk production. Therefore, the general health and defence status of the cow mainly determine the pathogenicity and clinical outcome of the disease (Burvenich *et al.*, 2003).

Initial bacterial load and multiplication within the udder are other important factors that have been hypothesised to play a role in the severity of *E. coli* bovine mastitis (Jones and Swisher, 2009; Almeida *et al.*, 2011). It was found that there is a difference in the multiplication rate of mastitic *E. coli* and normal environmental *E. coli*. The former is able to grow faster in the mammary gland (Almeida *et al.*, 2011). It was found that all mastitic *E. coli* isolates were fast growers in milk. On the other hand, environmental *E. coli* isolates are comprised of two groups, those being able to grow fast in milk and those who grow slower. This phenomenon could indicate that some *E. coli* strains are more susceptible to antimicrobial agents present in milk or they are less able to utilise the available nutrients in milk (Blum *et al.*, 2008).
The main source of mastitis causing *E. coli* is the intestinal flora of affected cows, other cows or calves. This indicates that contamination of the cow's environment with faeces could play a major role in the occurrence of *E. coli* mastitis (Green, 2002; Jones and Swisher, 2009). *E. coli* favours wet, warm and organic environmental conditions, which is similar to the conditions found within dairy herds (Winfield and Groisman, 2003). Findings from a UK study revealed that *E. coli* infections do not always behave in a constant manner. For example, over half of all *E. coli* mastitis cases were found to have originated from infections during the dry period of the cows, when teat sphincters were thought to be closed. However, not all cows infected during the dry period developed clinical mastitis (Green, 2002).

### 2.4 *E. coli* mastitis vaccines

Preventative control strategies are the most effective means to control mastitis development within dairy herds (Schroeder, 1997). Preventative control methods include i) good milking hygiene, ii) the use of properly functioning milking equipment, iii) provision of clean and dry housing conditions for cows, iv) sound nutritional programs and v) proper identification and treatment methods of cows that are infected with subclinical and clinical mastitis (Ruegg, 2001). The main purpose of preventative control methods is to reduce the number of bacteria to which the teat ends of cows are exposed to (Schroeder, 1997).

The biggest challenges in producing vaccines against *E. coli* mastitis are the vast range of *E. coli* strains that can cause mastitis, the genetic variation amongst these strains and the lack of correlation between virulence factors and the severity of mastitis (Silva *et al*., 2009; Suojala *et al*., 2011). Only a limited number of antigens can be included in a specific vaccine. It is impossible to cover the entire spectrum of *E. coli* mastitis pathogenesis in one universal vaccine. Vaccines can be effective in preventing recurrent *E. coli* infections, but is highly ineffective against new infections (Schroeder, 1997).

The main purpose of vaccination is the enhancement of the host’s immune response, but in the case of mastitis an enhanced immune response is not always beneficial (Chang *et al*., 2008). One component of immune response is the migration of large numbers of white blood cells to the infected gland. This leads to an increase of
somatic cells in the milk which reduces the quality of the milk. The nature of milk also hampers the effectiveness of vaccination (Talbot and Lacasse, 2005). The volume of milk present in the mammary gland dilutes the number of immune cells available to combat the infection and milk components such as fat and casein reduce the bactericidal abilities of the immune cells (Ruegg, 2001).

The success rates of mastitis vaccines may vary depending on the herd situation and conditions (Ruegg, 2001; Lee et al., 2005). It is expected of vaccines to reduce the severity and frequency of mastitis, prevent new infections and eliminate existing ones. Because of the complexity of *E. coli* mastitis, it is highly unlikely that one vaccine will achieve all of these objectives (Ruegg, 2001). Historically, vaccination against *E. coli* mastitis has not been successful. After vaccination, protective antibodies only appeared in serum but not in normal milk (Talbot and Lacasse, 2005).

The inner layer of the cell wall is common to various serotypes of *E. coli*. There is, however, a naturally occurring inner cell wall deficient *E. coli* J5 mutant that is currently used for vaccine production (Wilson et al., 2009). This vaccine appears not to prevent intramammary infection, but a reduction in clinical coliform mastitis incidence is evident in vaccinated herds (Denis et al., 2009). This, however, could lead to the persistence of high somatic cell count problems in vaccinated herds which are exposed to high *E. coli* challenges. A previous study also found that there is no difference in the rate of new infections between vaccinated and unvaccinated cows (Smith and Hogan, 1998).

New attempts have been made at designing more refined vaccines directed against *E. coli* mastitis, but effective results were limited. In a recent case the iron capturing structures of *E. coli* were used for vaccine antigens, but the vaccine failed to produce any antibodies directed against these antigens (Denis et al., 2009).
2.5 Methods for isolation, identification, antibiotic resistance determination, serotyping and genetic fingerprinting

2.5.1 Sampling, isolation and identification methods

The actual manner of clinical sample collection, storage and submission to the laboratory plays an essential role in the isolation and identification process of pathogens (Brenner et al., 2005). Poor practices in this area could lead to inaccurate results. Specimens must be collected aseptically in order to prevent environmental contamination and preferably from animals not treated with antimicrobial drugs, since these drugs could mask the cause of the disease. The stage of the disease at which samples are taken is also important. Sampling at the acute stages of the disease is desirable (Jarvis et al., 1994).

In the case of mastitis, milk samples are taken directly from infected cows showing clinical symptoms. The teat tip must first be thoroughly cleaned and then wiped with an aseptic solution such as sodium hypochlorite or 70% ethanol (Smith et al., 1985). Milk samples are then frozen until use. Milk naturally contains cryogenic protection substances which preserve microorganisms present in the milk when frozen (Bradley and Green, 2001). Milk samples must be transported in a cooled environment and allowed to thaw only once, before culturing and analysis commence (Smith et al., 1985).

Many simple agar media are available that can be used for the selective isolation of *E. coli* from clinical samples. These media contain substances that inhibit the growth of bacteria other than *Enterobacteriaceae*, such as tetrathionate, deoxycholate and bile salts (Brenner et al., 2005). The inhibition of microbial growth other than *Enterobacteriaceae* is of great importance since contaminants and other pathogens present in clinical samples may out compete and outgrow *E. coli* during the initial culturing stages (Jarvis et al., 1994).

Mastitis milk samples are first streaked onto trypticase soy agar plates containing 5% whole bovine blood and esculin. For the detection of coliform bacteria, milk from the sample is also streaked onto MacConkey agar plates. The plates must be incubated for 48 hours and examined after 24 and 48 hours (Smith et al., 1985).
*Enterobacteriaceae* are then tentatively identified and *E. coli* can then be identified by Gram-staining and various biochemical tests (Holt *et al.*, 2000; Singh and Prakash, 2008). Commercial testing kits utilizing biochemical reactions or antibody-antigen conjugation reactions coupled to rapid detection technologies (such as colour changes or fluorescence) have been developed. These kits can rapidly identify *E. coli* isolates from clinical samples (Brenner *et al.*, 2005).

MacConkey agar no. 3 is commonly used to distinguish *E. coli* from other *Enterobacteriaceae* since *E. coli* forms characteristic pink coloured colonies on this agar. The addition of 4-methylumbelliferyl-β-D-glucuronide (MUG) to MacConkey agar causes *E. coli* colonies to display fluorescence under UV light, which gives further confirmation of the positive identification of *E. coli* (Smith and Scotland, 1993).

Molecular techniques based on the polymerase chain reaction (PCR) are currently available and widely used for the identification of *E. coli*. This technique usually makes use of DNA probes targeting species specific sequences of the non-protein coding 16S rRNA gene. DNA probes targeting other species specific genes, O-antigen gene clusters or virulence factor genes have been identified and investigated extensively, which also aids in the accurate identification of *E. coli* (Brenner *et al.*, 2005; Lluque *et al.*, 2010; Wang *et al.*, 2010).

Real-Time PCR (rtPCR or qPCR) is another PCR based method which eliminates the need for gel electrophoresis after PCR cycling. Detection of PCR product formation is based on fluorescent technologies which are integrated into the thermal cycler and PCR reagents. The production of PCR products is also quantified after each cycle, which allows for quantification of template present in the original sample (Dorak, 2006; Dharmaraj, 2011). Methods for qPCR detection of certain *E. coli* strains in aqueous environments and dairy products have been described in literature (Lavender and Kinzelman, 2009; Singh *et al.*, 2009; Patel *et al.*, 2011).

### 2.5.2 Antibiotic resistance determination

Antibiotic resistance determination is becoming more essential in disease control management. Several tests are available for antibiotic resistance detection in bacteria. There is the standard disk diffusion method (Kirby-Bauer test) (Lindberg *et al.*, 1977)
as well as rapid detection methods such as molecular methods for the detection of antibiotic resistance genes and automated instruments utilizing photometric technologies. However, the disc diffusion method can, in addition to antibiotic resistance detection, also indicate the degree of resistance the bacterium possesses (Wise et al., 2002). The microdilution assay is another growth dependant assay which utilises 96-well plates. A dilution series of the antibiotic in question is made on the plate. Each well is then inoculated with a standardised inoculum of the microorganism in question. The growth in the wells is then recorded after the appropriate time of incubation (Arikan et al., 2002; Nascente et al., 2009).

The disk diffusion method is still the most widely used method for antibiotic resistance determination (Kahlmeter et al., 2009). This method simply entails complete spreading of a standardised bacterial pure culture over an appropriate agar plate, placing disks containing antibiotic substances in question on top of the culture on the agar plate and lastly incubating the agar plate under appropriate conditions before reading the results. Clear zones of no growth surrounding the antibiotic disks indicate sensitivity of the bacterium where the lack of clear zones indicates resistance. The length of the diameter of a clear zone can give a quantitative indication of the sensitivity to that specific antibiotic (Lacy et al., 2004; Hudzicki, 2010).

Disadvantages of the disk diffusion method are that it requires manual setup (which is time consuming) and interpretation which could give rise to inconsistent results (Hudzicki, 2010). The advantages are that it is inexpensive to perform, is widely used and is accepted in human and veterinary medicine. It is also readily adaptable to a variety of antibiotics and standardised commercial kits are available (Lacy et al., 2004).

2.5.3 Serological typing methods

Serological typing methods of bacteria are based on the presence of specific surface antigens a bacterium possesses and the variation amongst each of these specific antigens. This in turn affects the binding ability of antibodies directed against these antigens. The most important determinant in serotyping is the fact that, ideally, an antibody that can bind to one antigen variant must not be able to bind to other variants of the same antigen type (Robinson et al., 2010).
Serotyping of disease causing *E. coli* is mainly done on the O and H antigens (Holt *et al*., 2000). The most prominent difficulties with serotyping of *E. coli* are the large number of different O and H antigens present and cross reaction between different antigens. The procedure is also highly tedious and laborious to perform (Durso *et al*., 2005).

Because of the tediousness of the procedure and the vast number of O and H antigens present, serotyping of *E. coli* is becoming less feasible when studying and diagnosing the diseases it causes. Few laboratories possess the capabilities to perform substantial O and H antigen serotyping (Ballmer *et al*., 2007). In addition, cross reactivity between antigens and the inability to detect certain antigens are well known to render false results (Prager *et al*., 2003). In some cases it is documented that only a limited number of serotypes is known to cause a specific infection. Serotype screening for those specific strains only, may still be feasible and of value when studying the disease in question (Kausar *et al*., 2009; Lukjancenko *et al*., 2010). In the case of mastitis, however, there is no correlation between the O and H serotypical properties and the epidemiological and virulence characteristics of *E. coli* strains that cause the disease (Lipman *et al*., 1994).

New molecular methods that target the genes responsible for encoding the O and H antigens of *E. coli* have been developed (Durso *et al*., 2005; Ballmer *et al*., 2007). These methods are designed to detect variation within these genes in order to discriminate between serotypes or to identify only one specific serotype. Advantages of these methods are that they are faster and less laborious to perform, cross reactivity is decreased and certain antigens can be detected where traditional serological detection failed. Similar drawbacks, however, still remain. As with traditional serological methods, the molecular methods can only identify the specific serotypes that they were designed to detect. They are therefore limited to the number of serotypes they can detect. A larger number of serotypes require a larger number of oligonucleotide probes, which in turn could be more time consuming and lead to higher costs (Ballmer *et al*., 2007). The PCR-RFLP method could overcome this drawback. This method is based on the principle of one oligonucleotide primer pair that is used to amplify the entire gene in question of the strains. The amplified gene is then digested with a specific restriction enzyme in order to produce a characteristic restriction pattern. Variation and the subsequent serotype can be determined by
comparing the restriction pattern to an existing database of known restriction patterns (Aslani and Alikhani, 2009).

### 2.5.4 Genetic fingerprinting methods

Several molecular fingerprinting techniques have been developed in order to study genetic diversity and to do epidemiological typing of pathogenic bacteria. Most of these molecular techniques are based on PCR principles and restriction enzyme methodology (Radu et al., 2001). The target material is usually the genome of the pathogen. Genetic variation mainly results from neutral sites of DNA sequence variation within the genome and not necessarily within genes. These fingerprinting techniques all vary in their accuracy, reproducibility, cost, level of polymorphisms generated, time consumption and reliability (Van Belkum, 2002; Casarez et al., 2007).

#### 2.5.4.1 Random Amplified Polymorphic DNA

The random amplified polymorphic DNA (RAPD) method is a PCR based technique in which random unknown areas of the genome is amplified to produce several DNA fragments of different sizes (Dautle et al., 2002). Genomic DNA is the preferred template since the majority of the inherent properties of an organism are located on its genome. The RAPD method utilises a single arbitrary oligonucleotide primer of approximately 10 bases in length which binds to complementary sequences located randomly on the target DNA template (Mienie, 2003; Gomes et al., 2005). Some methods do employ the combined use of two to three 10-mer primers if no single primer is found to generate sufficient polymorphisms. The RAPD method is based on the assumption that the complementary DNA sequence of the primer will occur in the target genome on both DNA strands in opposite orientations within a distance from each other that is readily amplifiable by PCR (Mienie, 2003).

Polymorphisms in the RAPD banding patterns between isolates are mainly generated by three events. Base substitutions or deletions within the priming site that result in the presence or absence of bands at a specific locus. Insertions between two opposite priming sites that increase the distance between the priming sites beyond the amplifiable range, result in the disappearance of previous amplifiable fragments.
Lastly, insertions or deletions between two priming sites that significantly alter the size of the amplified fragment (Monna et al., 1994).

Cycling conditions of a RAPD reaction are similar to that of PCR. Primer annealing temperatures are usually considerably lower than that of conventional PCR because of the use of primers that consist of shorter sequences (Leuzzi et al., 2004). RAPD reactions generate several discrete DNA products and these products are considered to originate from different genetic loci in the genome. Amplified products are usually separated by agarose gel electrophoresis and are visualised on a UV transilluminator by using ethidium bromide staining. Analysis of RAPD banding patterns involves only the presence or absence of a band at a specific locus. This indicates that RAPD is a dominant marker and cannot be used to detect heterozygotes (Mienie, 2003).

Disadvantages of RAPD analysis are that inter- and intra-laboratory reproducibility is lower than with other molecular markers. The RAPD reaction is very sensitive to slight conditional changes (e.g. different thermal cyclers or polymerase enzymes used can yield different banding patterns). Furthermore, different genetic loci can produce similar DNA fragment sizes that cannot be distinguished by RAPD analysis (Bardacki, 2001; Ashbee and Bignell, 2010).

Advantages of RAPD analysis are that it is a quick, simple, low cost and efficient technique. No prior sequence information of the target genome is necessary for development. It requires small amounts of template DNA and it is reported that RAPD can detect higher levels of polymorphisms than other molecular markers (Vogel et al., 2000; Ashbee and Bignell, 2010). RAPD analysis has been widely tested on a variety of microorganisms with highly insightful results when compared to other molecular and traditional serotyping methods. RAPD analysis of pathogenic strains of *E. coli* has provided a deeper insight into their epidemiological nature due to RAPD’s high discriminatory power (Radu et al., 2001; Lin and Lin, 2007; Cagnacci et al., 2008).

### 2.5.4.2 Other molecular markers

Other molecular markers are available which can be applied for epidemiological and fingerprinting purposes. Examples of such methods are: Restriction Fragment Length Polymorphisms (RFLP), Simple Sequence Repeats (SSR), Amplified Fragment
Length Polymorphisms (AFLP), Multiple-Locus Variable Number of Tandem Repeat Analysis (MLVA), Enterobacterial Repetitive Intergenic Consensus sequence PCR (ERIC-PCR) and the \textit{E. coli} reference grouping PCR method (PCR ECOR) (Mienie, 2003).

The PCR ECOR method is a simple method that was developed targeting two known genes of \textit{E. coli} (the haem transport protein ChuA and the conserved stress-induced protein YjaA genes) and one anonymous DNA fragment. A triplex PCR is used to determine the presence or absence of these three loci in \textit{E. coli} isolates. This method is able to group an isolate into only one of four groups (group A, B1, B2 and D) based on the combination in which these three loci is present or absent (Clermont \textit{et al.}, 2000). Several studies have utilised this method and found to a certain extent some correlation between the ECOR groups and the pathogenicity and infection type of \textit{E. coli} isolates. However, this method cannot detect polymorphisms within each of its groups (Lai \textit{et al.}, 1999; Clermont \textit{et al.}, 2000; Watt \textit{et al.}, 2003).

The MLVA method targets genetic elements known as Variable Numbers of Tandem Repeats (VNTR’s). These elements are sequences of 10 to 100 bases in length that are organised in tandem repeats within genomes of organisms. Primer pairs are designed that flank the edges of specific VNTR loci in order to be amplified with PCR (Bustamante \textit{et al.}, 2010). Advantages of this method are it can detect satisfactory levels of polymorphisms, is highly reproducible between different laboratories and is relatively fast and simple to perform (Lindstedt \textit{et al.}, 2007; Heck, 2009). The main drawback is that extensive research is required for development. The location and sequences of VNTR loci within the genome of each individual species need to be predetermined and only a limited number of fixed loci can be utilised. Furthermore, although agarose gel electrophoresis is sufficient for fragment sizing analysis, highly expensive capillary electrophoresis is the preferred method for fragment size analysis in order to increase the discriminatory capabilities and reproducibility of the method (Olsen \textit{et al.}, 2009; Schouls \textit{et al.}, 2009).

ERIC sequences are similar to repetitive extragenic palindromic (REP) sequences and BOX elements. All three elements are repetitive sequences which are highly conserved and randomly dispersed throughout the genome (Zulkifli \textit{et al.}, 2009). Primers are designed targeting ERIC sequences and variation of the DNA located
between ERIC loci give rise to polymorphic banding patterns between different isolates. As with MLVA methods, known sequence data is required for the development of this technique. Lower discriminatory power between *E. coli* isolates using ERIC-PCR has also been reported (Casarez *et al*., 2007; Duan *et al*., 2009).

The SSR analysis method is a PCR based technique that targets microsatellites occurring in the genome. Microsatellites are tandem repeats of sequence units generally less than 5 base pairs in length and are also distributed throughout the genome. They are thought to be produced by errors during DNA replication (Gur-Arie *et al*., 2000). SSR primers target specific areas in the genome containing microsatellites and flank the microsatellite sequence (Qosim *et al*., 2011). The main disadvantage of SSR analysis is that the development of the technique is extremely time consuming and laborious. It involves fragmentation of the genomic DNA with restriction enzymes, cloning of the fragments into plasmids, screening the cloned fragments for microsatellite repeat regions, sequencing of the positive clones and then finally designing new specific primers flanking the repeat region. Advantages of SSR analysis includes production of large numbers of polymorphisms, inter-laboratory reproducibility is highly accurate and a developed technique for a specific organism can in most cases be applied directly to close related species (Mienie, 2003; Mrazek *et al*., 2007; Qosim *et al*., 2011).

The AFLP analysis method utilises both restriction enzyme digestion and PCR methodology (Riley and Liu, 2007). The genomic DNA is firstly digested by restriction enzymes (usually 2 enzymes are used simultaneously). The next step involves the addition of dsDNA adapters to the restriction fragments. Finally these fragments are subjected to PCR cycling using primers complimentary to the adapters at their 5’ ends, but also contain up to three additional random bases at their 3’ ends. These random bases at the 3’ ends of the primers allow for selective amplification of the restriction fragments (Xu *et al*., 2000; Chial, 2008).

The disadvantages of AFLP are that it involves more than one step which makes it more laborious than other PCR-based methods. It cannot distinguish between hetero- and homozygotes. Scoring of the generated bands is complex and the procedures are technically demanding to perform. The advantages of this technique are that it produces large numbers of polymorphisms, no prior sequence information is required,
it is highly reproducible and there are standard commercial kits available. (Mueller and Wolfenbarger, 1999; Chial, 2008).

2.5.4.3 Phylogenetic tree construction

The most commonly used statistical algorithm for the construction of phylogenetic trees from RAPD banding patterns is the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). This is the simplest method for genetic distance tree construction. It employs a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is build in a stepwise manner (Opperdoes, 1997).

UPGMA assumes that the rates of evolution are approximately constant among different lineages. First the two closest related operational taxonomic units (OTUs) are identified and are then treated as a single OTU. Such an OTU is referred to as a composite OTU. Subsequently the next closest related OTU to this new composite OTU is identified and paired with it. This process continues until there are only two OTUs left (Backeljau et al., 1996).

Phylogenetic trees constructed by UPGMA analysis provides a clear visual presentation of the genetic relatedness between the organisms that were analysed (Thomas, 2002). The degree of differences in RAPD banding patterns between organisms are depicted as percentages at each split in the horizontal tree line. Therefore close related and highly distant organisms are clearly visualised on the tree with the percentages of difference between them indicated (Carr, 2007).

2.6 Autogenous vaccines

Autogenous vaccines are vaccines that are produced mainly from inactivated whole cells of pathogenic bacteria (Meulemans et al., 2011). The pathogens used for vaccine production originated from the individual itself for whom the vaccine is intended. Autogenous vaccines are considered to be an old technology and have been utilised for several decades (Lapointe et al., 2002). Further developments resulted in autogenous vaccines that were formulated utilising specific antigens only
(not whole inactivated cells). The production of viral autogenous vaccines has also been reported (Turk et al., 2005).

These vaccines are mainly produced for the specific region the pathogens originated from and are therefore farm specific (Lapointe et al., 2002). The main difficulty arising when following such a formulation strategy is when there are a large number of pathogens initially isolated and/or the number of pathogens increase over time. There is a limit to the number of pathogens that can be included per vaccine (approximately a maximum of 8 isolates per vaccine). If there are too many different antigens present in a specific vaccine, the animals will not be able to produce protective levels of antibodies against all of the antigens. This could result in the absence of certain antibodies together with insufficient levels of the remainder of the antibodies. For these reasons a sufficient screening method needs to be employed for the selection of appropriate isolates to be included in the vaccine in order for the vaccine to be effective (Wiltz et al., 1995).

Pathogenic bacteria used for autogenous vaccine production are mainly inactivated with formalin. The vaccine is administered intramuscularly, subcutaneously or intranasal (Lapointe et al., 2002). Autogenous vaccines have been produced from various pathogenic bacteria directed against various types of human and animal diseases (Wiltz et al., 1995). A few cases of inconsistencies and ineffectiveness in vaccine efficiency have been reported. This can be attributed to the loss of antigenic properties due to formalin treatment or production of antibodies directed against antigens not associated with the virulence factors (Lapointe et al., 2002; Denis et al., 2009; Funk et al., 2009). There are, however, also reported cases of highly efficient autogenous vaccines that eliminated persistent diseases completely from the affected farm (Gurung et al., 2006; Thaiya et al., 2010; Wambura, 2010).

There is a growing trend in European Union countries towards the use of autogenous vaccines for disease control in commercial farming (Hera and Bures, 2004). The market for registered vaccines is changing significantly due to the declining number of new emerging diseases. Resources and time required for the registration of new vaccines are continuously increasing and the high economic risk results in the withdrawals of new vaccines from niche markets (Von Hankó, 2009). Mutations and resistance are emerging amongst present pathogens. There is also variability within
new emerging diseases and the practices and priorities of farm management (e.g. free range farming and cost pressure) are changing (Cooper, 2010a). These are all factors giving rise to a higher need for flexible solutions in disease control (Cooper, 2010b). Autogenous vaccines can fulfil this need and is becoming an integral part of modern animal health. This is because of the fact that autogenous vaccines are efficient, can be used in the case of non-availability of registered vaccines and the changed priorities of registered vaccine producers. However, great emphasis must be put on the avoidance of unregulated and non-harmonised autogenous vaccines (Von Hanko, 2009).

2.7 Summary of literature

The preceding literature review has demonstrated that mastitis is still a major problem faced by the dairy industry today. Mastitis leads to severe financial losses due to a reduction in milk quality and the quantity of milk production. It can also affect the general health condition of dairy herds. Loss of appetite, dehydration, rapid pulse, high fever and death may occur.

Literature cited has also shown that mastitis is a very complex disease which can be caused by a variety of bacteria. The mastitis pathogens are categorised into two main groups, namely contagious pathogens and environmental pathogens. The contagious pathogens were considered to be the dominant cause of mastitis, but over the past few years the environmental pathogens became the prominent problem in dairy herds. *E. coli* has been identified as one of the major environmental pathogens causing both clinical and subclinical mastitis. Mastitis caused by *E. coli* is very difficult to control within dairy herds. The emergence of various multidrug resistant strains and the high levels of genetic variability within the species have rendered antibiotic treatments and vaccinations costly and ineffective.

Various studies have utilised and compared different genotyping methods of *E. coli* to give insight into the epidemiological nature of this pathogen. RAPD analysis was identified as one of the efficient methods. Compared to other methods this method is simpler and less laborious to develop and to be implemented. The discriminatory
capabilities of RAPD analysis is higher than with most other methods, it is fast and cost effective and it does not require the use of sophisticated expensive equipment.

The literature review further revealed that antibiotic resistance determination and proper genotyping analysis of pathogenic *E. coli* could greatly aid in the understanding and control of diseases caused by *E. coli*. The use of autogenous vaccines is becoming a more important part of disease control in animals. There are less new emerging diseases and mutations and resistance in the pathogens causing existing diseases are rendering registered vaccines less effective. There is a growing need for adaptable, efficient vaccines for current diseases. Autogenous vaccines are believed to fulfil this need and must become an integral part in the future of disease control.
CHAPTER 3
MATERIALS AND METHODS

3.1 Study site and sampling

A dairy farm housing approximately 1 000 Holstein cows in the Darling area, Western Cape Province of South Africa, was selected for this study. Sampling was conducted in two phases. Phase 1 was from August 2004 to October 2004 (to establish background data prior to vaccinations). Phase 2 was from May 2005 to December 2007. All cows showing clinical symptoms of mastitis were identified and sampled. In random cases, cows with no clinical signs of mastitis were also sampled for bacteriological analysis. Milk samples were taken directly from the udders of the cows by the farmer or his employees into sterile test tubes. The tubes were then frozen and stored until shipment. Tubes were shipped once or twice per month, on ice, to Disease Control Africa (DCA), Pretoria. All sample logistics were managed by Disease Control Africa.

3.2 Isolation, identification and antibiotic resistance testing of *E. coli*

Milk samples were sent from Disease Control Africa to Golden Vet Labs (Kyalami, Gauteng) for analysis using standard bacteriological procedures (Jarvis *et al.*, 1994). The milk samples were plated out on Sheep blood agar and MacConkey agar plates. Colony morphology and colour on MacConkey agar plates together with the Gram stain procedure were used as initial identification of *E. coli* colonies. Standard biochemical tests were used as confirmation of identification (Jarvis *et al.*, 1994; Brenner *et al.*, 2005). Since *E. coli* is not a natural inhabitant of the udder (Suojala *et al.*, 2011), it was assumed that it is unlikely for more than one strain to infect a quarter simultaneously. Therefore one *E. coli* colony per milk sample was used to generate a pure culture which was grown on either a nutrient agar or MacConkey agar plate. These pure cultures were sent to Disease Control Africa where they were used for molecular analysis and autogenous vaccine production. Each *E. coli* pure culture was linked to its original milk sample number from which it was isolated and this number was used for identification purposes in the downstream processes. Antibiotic
resistance testing was done by Disease Control Africa and Golden Vet Labs using the 
disk diffusion method (Atlas, 1997). The following antibiotics were used in the test: 
Ampicillin (10µg), Cefuroxime 2 (30µg), Cloxacillin (1µg), Lincomycin (2µg), Neomycin 
(30µg), Novobiocin (30µg), Polymyxin B (300 units), Streptomycin (10µg), Tetracycline 
(30µg) and Cephalothin 1 (30µg). An antibiotic resistance variance tree was 
constructed from the antibiogram data. Each antibiotic represented a locus and the 
variance at each locus was scored as 1 for sensitive, 2 for intermediate and 3 for 
resistant (Hudzicki, 2010). The tree was constructed with the Tools For Population 
Genetic Analysis software (TFPGA, v1.3, Miller, Northern Arizona University, USA) 
package using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) 
statistical algorithm.

3.3 Genomic DNA isolation

A single colony was scraped from each agar plate and inoculated into 25ml sterile 
nutrient broth in a 100ml Erlenmeyer flask. The broth was incubated overnight at 37°C 
in a shaker incubator at 150rpm. The DNA extraction method of Marmur (1961) was 
modified and used to extract genomic DNA from the broth cultures. Of the overnight 
broth cultures, 14ml were poured into 15ml centrifuge tubes. The tubes were 
centrifuged at 3500rpm for 10 min in a bench-top centrifuge (Hettich EBA 8S, 
Germany) to pellet the cells. The supernatants were discarded and the cell pellets 
were re-suspended in 1.2ml cell suspension buffer (10mM Tris-HCl pH 8.0, 1mM 
EDTA, 0.35M Sucrose) by shaking and vortexing the tubes. Following complete re- 
suspension of the cells, 20µl Lysozyme solution (5mg/ml) was added to the 
suspension and the tubes were incubated at 37°C for 30 min in a water bath. After 
incubation 1.2ml lysising solution (100mM Tris-HCl pH 8.0, 20mM EDTA, 0.3M NaCl, 
2% SDS), 1ml NaClO₄ (5M) and 10µl Proteinase K solution (20mg/ml) were added to 
the tubes. The tubes were inverted 5 times to mix the contents and incubated for 1 h 
at 55°C in a water bath. An equal volume of Phenol:Chloroform:Iso-Amyl alcohol 
(25:24:1) solution were added to the tubes. The tubes were shaken vigorously by 
hand to mix and then placed horizontally on a shaker for 20 min at 250rpm. The tubes 
were shaken by hand at 5 min intervals during the shaking time. Afterwards the tubes 
were centrifuged at 4000rpm for 20 min. Upper aqueous phases of each tube were 
transferred to new 15ml centrifuge tubes. The Phenol:Chloroform:Iso-Amyl alcohol 
extrac
aqueous phases after the second extraction were again transferred to new tubes and overlaid with 0.6 volumes of ice cold Iso-propanol (100%). The tubes were then swirled upright in a circular motion until DNA pellets precipitated in the solutions. Precipitated DNA pellets were removed with Bibby pipettes and transferred to 1.5ml microcentrifuge tubes. The microcentrifuge tubes were centrifuged at 6000rpm for 30 sec in a bench-top centrifuge (Hettich Mikro 20, Germany) to pellet the DNA. Supernatants were discarded. Then, 1ml 76% ethanol was added to the tubes and the tubes were incubated at room temperature for 10 min. The ethanol was discarded and the DNA pellets were dried by placing the tubes with their caps open in a dry oven for 15 min at 30°C. Tubes were checked at 5 min intervals in order to prevent over drying of the DNA pellets. Dried DNA pellets were dissolved by adding 150µl TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) and 2µl RNase A solution (50 units RNase A /ml, 50% glycerol, 10mM Tris-HCl pH 8.0) to the tubes and incubating the tubes at 4°C overnight. If the DNA pellets were visually dissolved in a shorter period of time, the procedure was continued without overnight incubation. After the DNA pellets have completely dissolved, the tubes were incubated at 37°C for 1 h in a water bath to digest RNA contamination. Next, 0.1 volumes of 3M Sodium Acetate solution (pH 4.6) was added to the tubes and the tubes were vortexed. The solutions in the tubes were overlaid with 2.5 volumes 95% ethanol and the tubes were centrifuged at 6000rpm for 20 min to precipitate the DNA. The supernatants were discarded and 1ml 76% ethanol were added to the DNA pellets in the tubes and incubated at room temperature for 10 min. The ethanol was discarded and the DNA pellets were dried for 15 min at 30°C in a dry oven, similar to the drying procedure described above. The DNA pellets were re-suspended in 100µl TE buffer and allowed to dissolve in the fridge at approximately 4°C. The tubes were stored in the fridge until required.

### 3.4 DNA quality and quantity determination

The quality and quantity of the extracted genomic DNA was determined using a spectrophotometric analysis method. A WPA Lightwave UV/Visible spectrophotometer (WPA, UK) was used. Each DNA sample was diluted 10x (45µl molecular grade water and 5µl DNA solution) and readings were taken at 260nm and 280nm wavelengths. Three readings at both wavelengths were taken for each sample and the average reading was calculated. The 260:280 ratios were subsequently used to calculate the
purity of the DNA. The DNA concentrations (ng/µl) were determined by using the following equation:

\[ [\text{DNA}] \text{ ng/µl} = \text{OD } 260\text{nm} \times 50\text{ng/µl} \times 10 \text{ (dilution factor)} \]

3.5 RAPD development

The full genome sequence of *E. coli* K-12 (Accession no: NC_000913) were downloaded from the NCBI PubMed Nucleotide website [http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide](http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) and loaded into pDRAW analysis software (v 1.1.76, AcaClone Software). Thirty two random 10-mer oligonucleotide primers were derived directly from various sequences throughout the genome. These primers were screened for priming sites using pDRAW. Four primers (Table 4.1) showing satisfactory multiple annealing sites on the genome were selected for the RAPD reaction development. The annealing temperatures were optimized using Oligo Explorer Analysis Software (v 1.1.2, Gene Link) and by performing RAPD reactions using the genomic DNA isolated from two random *E. coli* isolates.

The 2 primers that each generated the largest number of bands during the initial annealing temperature optimization experiments were selected for PCR buffer optimization. A PCR buffer optimization kit from Sigma Aldrich, SA, (PCR Optimization Kit II) was used for the buffer composition optimization. Finally 1 primer (EcRAPD4) that generated the largest number of DNA fragments was selected for the RAPD analysis. This primer was tested on 20 random *E. coli* isolates for its efficiency and reproducibility.

3.6 RAPD reaction conditions

The RAPD reactions were done by using the GeneAmp PCR System 2700 (Applied Biosystems, USA) thermal cycler. The final volume for each reaction was 50µl, consisting of 33.5µl of PCR water (Sigma Aldrich, Germany), 5µl of 10x RAPD buffer (104mM Tris-HCl pH 9.0, 35mM MgCl₂, 250mM KCl), 1µl of deoxynucleotide mix (10mM each of dATP, dCTP, dGTP, dTTP, Sigma Aldrich, Germany), 4µl of EcRAPD4 primer (5’-CGCTGGCAGCG-3’, 5µM, Inqaba Biotech, RSA), 4µl of genomic DNA template (200ng to 500ng in total) and 2.5µl of DNA polymerase (JumpStart
RED Taq DNA Polymerase, Sigma Aldrich, Germany). The tubes were placed into the GeneAmp PCR System 2700 which was programmed according to the following parameters: initial denaturation for 5 min at 95°C, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 2 min and extension at 72°C for 2 min. The 45 cycles were followed by a final extension step at 72°C for 10 min. The tubes were subsequently stored in the fridge at 4°C until further use.

3.7 Agarose gel electrophoresis

The polymorphic DNA fragments generated by the RAPD reactions were separated by using agarose gel electrophoresis. Agarose gels of 1.8% (w/v) containing ethidium bromide (1µg/ml) were used. A volume of 10µl of each RAPD reaction solution was loaded directly into each well of the agarose gel. The JumpStart RED Taq DNA polymerase solution contains a red loading dye, therefore no additional loading dye was required for electrophoresis. A DNA molecular weight marker (DirectLoad Wide Range DNA Marker, Sigma Aldrich, Germany) was used to determine the DNA fragment sizes generated by the RAPD reactions. The gels were run for approximately 3 h at 45V (until the dye was +/- 1cm from the edge of the gel) to achieve DNA fragment separation. The gel images were captured by using a GelDoc-It Imaging System and Labworks Image Acquisition and Analysis software (v1.1.2, UVP, UK). The UV lamp was set at a wave length of 302nm.

3.8 Gel analysis and phylogenetic tree construction

The DNA band patterns on the captured agarose gel images were quantified and analysed by using Labworks Image Acquisition and Analysis software (v1.1.2, UVP, UK). The data containing the DNA fragment sizes were exported from Labworks to Excel (Microsoft, USA) in table format. The alignment and scoring of the DNA bands were done in Excel, with each column representing the banding pattern generated from each *E. coli* isolate and each row representing a locus of DNA fragments with similar sizes. DNA fragments that differed by approximately 50 base pairs or less were considered to be of similar sizes and therefore located at the same locus. However, due to the less precise fragment sizing nature of horizontal agarose gel electrophoresis and sizing variance between different gels, greater lenience in
fragment size differences were allowed where deemed necessary. The absence of a band at a specific locus was scored with a 1 and the presence of a band was scored with a 2. After scoring, the data was used for the phylogenetic tree construction. The phylogenetic trees were constructed by using TFPGA software (v1.3, Miller, Northern Arizona University, USA). The data was imported from Microsoft Excel into TFPGA and the phylogenetic trees were constructed based on the genetic distances between the isolates by using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) statistical algorithm.

3.9. Vaccine production and administration

The autogenous vaccines were produced at the facilities of Disease Control Africa (Pretoria, RSA). A single colony from a pure culture was used to inoculate 150ml Nutrient broth in a 250ml Erlenmeyer flask. The broth culture was incubated overnight at 37°C in a shaker incubator at 150rpm. Cells were harvested by centrifugation for 10 min at 4500rpm. The supernatant was discarded and the cell pellet was re-suspended in 400ml saline (0.9% NaCl) in a 500ml media bottle. A series dilution was made from the cell suspension (Jarvis et al., 1994). The cell count was done on the 10^-2 dilution by using a Neubauer counting chamber (Caprette, 2000). A volume of 3.5ml formalin (36%) was added to the cell suspension and the bottle was incubated overnight at room temperature on a shaker at 150rpm. The inactivated cell suspensions were used to produce the vaccines. The vaccines were made up with saline containing 9 x 10^8 cells/ml of each isolate and a unique DCA immune stimulant.

The *E. coli* isolates to be included in the vaccines were chosen based on the RAPD generated phylogenetic trees. The general criteria that were adhered to for autogenous vaccine formulation from the RAPD results were: 1) if two or more *E. coli* isolates showed a genetic distance of 15% or less amongst each other, only one isolate from that group is selected to be included in the vaccine. 2) As the number of *E. coli* isolates received increases over time and the previous vaccine strains have genetic distances of 20% or higher in comparison to the new isolates, those previous strains will be excluded from the new vaccine and replaced with new isolates. However, due to variances in the number of monthly isolates and the occasional need to include other organisms in the vaccines, these criteria were used as guidelines only and in some cases a certain degree of deviance had to be allowed upon vaccine
formulation. One vaccine was formulated using the antibiogram variance tree with similar selection criteria as with the RAPD based formulation.

The vaccines were shipped to the farm where the farm employees administered the vaccines to the entire herd. They were administered nasally at dosages of 2 ml per animal. Each vaccination consisted of 2 applications, a primary application followed by a booster application approximately one to two weeks after the primary application. The vaccines were re-formulated with new emerging \textit{E. coli} isolates throughout the study period.

3.10 On farm mastitis monitoring

The entire lactating herd was monitored for mastitis during each milking and the numbers of such cases were documented daily for a period of 48 months (January 2005 to December 2008). This included the period of 5 months prior to the first vaccination and 12 months after the last vaccination. The daily numbers of cows that died or were slaughtered as a result of any disease (including mastitis) were included in the monitoring programme. Mastitis monitoring was done on-site by the farm employees by physical inspection of the udders and by using the standard strip cup test. This test entails squirting milk directly from the teat through a fine sieve into a cup. The presence of visible flocculates on the sieve is an indication of mastitis.

3.11. Statistical Analysis

Statistical analysis was done on the number of recorded mastitis cases and the number of \textit{E. coli} isolates recovered during the study period. The two data sets were each split into two arrays. Array one consisted of the data collected before the first vaccination (May 2005 and earlier) combined with the data collected after the last vaccination (January 2008 to December 2008). Array two consisted of the data collected during the entire vaccination period (June 2005 to December 2007). The P-values between the two arrays of each data set were calculated with the parameters set as \textit{tails} = 1 and \textit{type} = 3. The P-value between the \textit{E. coli} isolates (array 1) and all the other isolates recovered (array 2) during the study period was also calculated using the same parameters as mentioned above.
CHAPTER 4

RESULTS

4.1 Mastitis occurrence, samples and pathogen isolates

During the 48 month period of mastitis monitoring on the farm, the lactating herd consisted on average of approximately 1000 cows. A total of 1 125 cases of clinical mastitis were reported, 168 cows died and 446 cows were slaughtered. Although the deaths and slaughtering were a result of various illnesses, severe clinical mastitis made a contribution to the number of cows that were slaughtered.

Figure 4.1: A graph illustrating the monthly numbers of lactating cows bearing clinical mastitis as well as the number of cows that died or were slaughtered each month.

The general health status of the dairy herd is illustrated in Figure 4.1. This figure depicts the monthly numbers of cows with clinical mastitis symptoms, cows that were slaughtered and cows that died. The graph can be divided into three clear time frames based on the illustrated data; 1) January 2005 to August 2005, 2) September 2005 to March 2008 and 3) April 2008 to December 2008. Time frames 1 and 3 had significant higher incidences of mastitis cases within the herd than time frame 2 (P=0.0025).
Time frame 2 coincides almost directly with the entire vaccination period of the herd. Vaccination started in June 2005 and was applied at continuous intervals to the herd until mid December 2007. This trend in clinical mastitis cases gives a strong indication that the mastitis vaccines have greatly reduced and controlled the occurrence of mastitis within the herd.

Over the 41 month sampling period, a total of 921 milk samples were collected from the dairy farm and sent to Golden Vet Laboratories. Cows bearing clinical mastitis as well as randomly selected healthy cows were included in the sampling. All milk samples were screened for the common mastitis causing pathogens (mastitigens), resulting in the isolation of 349 mastitigens in total. A total of 250 samples exuded contaminant growth of no significance and 322 samples exhibited no growth. The numbers of each mastitigen isolated are depicted in Figure 4.2.

For logistical reasons no samples were sent in by the farm for analysis from November 2004 to April 2005, April 2007 and from June 2007 to August 2007, which accounts for the zero figures in the data series of Figure 4.2.

Figure 4.2: A graph illustrating the monthly number of mastitis causing pathogens isolated during the sampling period.

The group of mastitigens were comprised of 181 *E. coli*, 18 *Streptococcus uberis*, 46 *Streptococcus dysgalactiae*, 1 *Streptococcus agalactiae*, 21 *Staphylococcus*
epidermidis, 13 Archanobacterium pyogenes and 69 other bacterial isolates (including Enterococcus sp., Citrobacter sp., Enterobacter sp. and Klebsiella sp. which in rare cases could cause mastitis). The contribution of each of the above mentioned pathogens to the total number of mastitisogens are depicted as percentages in Figure 4.3.

![Figure 4.3](image)

**Figure 4.3:** The total mastitisogen composition of all the pathogens that were isolated throughout the sampling period.

In this case study, initial sampling was conducted for a period of three months, from August 2004 to October 2004, prior to any mastitis vaccinations. The data of the mastitisogen isolates recovered during this period depicted in Figure 4.2 clearly indicates that *E. coli* was, by a considerable margin, the major cause of clinical mastitis within the herd. During all three these initial months, *E. coli* accounted for more than 60% of the total mastitisogens isolated. This initial mastitis pathogen isolation data formed the premise for choosing this specific herd for the *E. coli* mastitis case study. Figure 4.2 further indicates that *E. coli* remained the dominant cause of mastitis within the herd even during periods with lower numbers of mastitisogens isolated. Figure 4.3 illustrates that *E. coli* accounted for 51.9% of the total number of pathogens isolated throughout the entire sampling period. The second highest contributor was the group of other
bacteria with only 19.8%, which is substantially less than the *E. coli* isolates. The statistical analysis further confirmed that the number of *E. coli* isolates recovered during the sampling period were significantly higher than all the other pathogens recovered ($P=2.44 \times 10^{-7}$).

**Figure 4.4:** The total number of *E. coli* isolates isolated each month and the trend line indicating the average number of isolates over every 2 months. The vaccines administered each month are also indicated below the months.

The data in Figures 4.2 and 4.4 indicate that December 2006 yielded only 4 *E. coli* isolates. November 2006 yielded 12 isolates and January 2007 yielded a high number again of 14 isolates. The indication of a reduction in *E. coli* mastitis cases during December 2006, however, is inaccurate for this period. Due to the backlog created when RAPD analysis was resumed in mid December 2006 (see section 4.3), many of the samples taken in December 2006 were retained on the farm and sent to DCA in January 2007 for analysis. Unfortunately, these samples were sent together in one batch with the samples of January 2007 without any indication of which samples were taken in December and which in January. Therefore, the trend line added in Figure 4.4 gives a more accurate representation of the isolate numbers from November 2006 to January 2007. This was the only incidence of sample retention during the entire study.
period and did not have an influence on the true representation and interpretation of the results. The trend line in Figure 4.4 further illustrated a higher occurrence of *E. coli* mastitis before vaccination started than during the vaccination period. Statistically, the numbers of *E. coli* isolates recovered prior to the vaccination period were significantly higher than during the vaccination period (*P*=0.0023).

![Figure 4.5](image)

*Figure 4.5:* The monthly numbers of the total mastitigens isolated are depicted as percentages of the number of milk samples taken each month.

The percentages of total mastitigens isolated each month (calculated from the total monthly number of samples taken and analysed) are depicted in Figure 4.5. The highest percentage of mastitigens isolated from the samples was 79% in August 2004, and for the vast majority of months the mastitigens isolated were below 60%. These findings indicate that the number of samples taken during the sampling period were not a limiting factor in the numbers of mastitigens isolated. It can therefore be concluded that these mastitigens isolated were an accurate reflection of the pathogens present within the herd during the sampling period.

### 4.2 Antibiotic resistance amongst *E. coli* isolates

The antibiotic resistance profiles of 145 *E. coli* isolates, which represented the entire sampling period, were determined. Each *E. coli* isolate was tested against 10
antibiotics and the susceptibility of all the 145 isolates to each antibiotic are depicted as percentages in Figure 4.6. For example with Ampicillin, 26% of the isolates were sensitive, 43% were intermediate and 31% were resistant to the antibiotic. The average percentages of sensitive, intermediate and resistant isolates of all the antibiotics combined are included in Figure 4.6. The majority of the isolates were sensitive to Cefuroxime 2 and Polymyxin B (68% and 63%, respectively). In general, many of the isolates were resistant to several antibiotics. Virtually all isolates were resistant to Cloxacillin, Lincomycin, and Novobiocin (100%, 99%, and 98%, respectively). Furthermore, 70% of the isolates were resistant to Neomycin.

Figure 4.6: The susceptibility patterns of all the E. coli isolates combined to each antibiotic as well as the averages of all antibiotics combined.

None of the E. coli isolates were sensitive to all the antibiotics and only one isolate was resistant to only two antibiotics. All the other isolates were resistant to at least three or more antibiotics. The dominant multidrug resistant pattern was Cloxacillin, Lincomycin, Neomycin and Novobiocin (CLNN pattern). This pattern occurred in 69% of the isolates. The antibiogram data of the selected 100 isolates (Section 4.3) derived from the disk diffusion method is depicted in Appendix A.
4.3 RAPD analysis, phylogenetic and antibiogram variance

The four primers that were selected for RAPD development generated between 0 and 4 bands when they were individually tested on the two random *E. coli* isolates. The sequences of these primers are depicted in Table 4.1. Primers EcRAPD1 and EcRAPD4 generated the most bands (2 and 4 respectively) during the annealing temperature optimization stage and were subsequently used for PCR buffer optimization. During the PCR buffer optimization stage primer EcRAPD1 generated only a maximum of 4 bands where primer EcRAPD4 generated a maximum of 11 bands. Therefore, primer EcRAPD4 was selected for RAPD analysis of the *E. coli* isolates.

Table 4.1: The sequences of the four primers that were selected to undergo RAPD reaction development.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcRAPD1</td>
<td>5'-CCGCCCGGCG-3'</td>
</tr>
<tr>
<td>EcRAPD2</td>
<td>5'-CCGCTGGCCG-3'</td>
</tr>
<tr>
<td>EcRAPD3</td>
<td>5'-GGCGGCGCGC-3'</td>
</tr>
<tr>
<td>EcRAPD4</td>
<td>5'-CGCTGGCCGCG-3'</td>
</tr>
</tbody>
</table>

RAPD analysis was done on the *E. coli* isolates as they were received by DCA each month. The results of each new batch of isolates were then integrated with the results of all the previous isolates and each time a new phylogenetic tree was constructed. As a result, several phylogenetic trees were constructed during the study period. The newly constructed trees served as updated versions of the previous trees. The genetic variances amongst the newly added isolates as well as their relation to the previous isolates were illustrated in the new trees. From March 2006 up to December 2006 no RAPD analysis was done on the incoming *E. coli* isolates due to the relocation of the molecular laboratory. From mid December 2006 RAPD analysis was resumed and the isolates received from March to December 2006 were analysed and integrated with the previous results. Due to financial restrictions RAPD analysis was not done on all
181 *E. coli* isolates recovered during the study. After periods that rendered high numbers of *E. coli* isolates, certain isolates were randomly selected to be left out of RAPD analysis. As a result RAPD analysis was done on 113 isolates (Table 4.2).

The numeric analysis of the RAPD raw data and polymorphisms generated are depicted in Table 4.2. The RAPD reactions generated highly polymorphic banding patterns between the *E. coli* isolates with generally large numbers of DNA fragments produced from each isolate. The total number polymorphic bands generated accounted for 68% of the total bands scored. However, the percentages of polymorphic bands from the different time periods ranged from 41% to as high as 90%. This gives a strong indication that the present RAPD method possesses high discriminatory capabilities, but is still able to indentify close related strains (Table 4.2; Figure 4.9).

An average of 10 DNA fragments per isolate were generated with the RAPD analysis. The lowest number of fragments produced was 5 fragments which occurred in only 2 isolates (isolates 85 & 89; Table 4.3). RAPD fragment sizes ranged in total between approximately 4700 base pairs (bp) and 170bp. Table 4.2 further indicates that the fragment size ranges of the different time periods were constantly broad, which in turn could have facilitated proper discrimination on a continuous bases. Four of the RAPD fragments (sizes approximately 650bp, 1050bp, 1350bp and 1450bp; Figure 4.7) occurred in random combinations in the majority of the isolates (≥87%).
Table 4.2: The numeric analysis of the RAPD raw data extracted from the agarose gels.

<table>
<thead>
<tr>
<th>Period</th>
<th>Number of isolates</th>
<th>Total number of scored bands</th>
<th>Polymorphic bands</th>
<th>Polymorphic bands %</th>
<th>Bands size range (kb)</th>
<th>Average no. of bands per isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>September to October 2004</td>
<td>23</td>
<td>225</td>
<td>156</td>
<td>69%</td>
<td>0.24 - 4.69</td>
<td>9.78</td>
</tr>
<tr>
<td>May to July 2005</td>
<td>6</td>
<td>65</td>
<td>29</td>
<td>45%</td>
<td>0.24 - 2.83</td>
<td>10.83</td>
</tr>
<tr>
<td>October to November 2005</td>
<td>9</td>
<td>86</td>
<td>59</td>
<td>69%</td>
<td>0.24 - 2.50</td>
<td>9.56</td>
</tr>
<tr>
<td>January to February 2006</td>
<td>8</td>
<td>72</td>
<td>40</td>
<td>56%</td>
<td>0.17 - 3.44</td>
<td>9</td>
</tr>
<tr>
<td>May to June 2006</td>
<td>11</td>
<td>132</td>
<td>110</td>
<td>83%</td>
<td>0.19 - 4.62</td>
<td>12</td>
</tr>
<tr>
<td>July to August 2006</td>
<td>8</td>
<td>61</td>
<td>29</td>
<td>48%</td>
<td>0.63 - 3.41</td>
<td>7.63</td>
</tr>
<tr>
<td>September to October 2006</td>
<td>10</td>
<td>96</td>
<td>86</td>
<td>90%</td>
<td>0.24 - 2.87</td>
<td>9.6</td>
</tr>
<tr>
<td>November to December 2006</td>
<td>12</td>
<td>134</td>
<td>86</td>
<td>64%</td>
<td>0.25 - 3.43</td>
<td>11.17</td>
</tr>
<tr>
<td>January to March 2007</td>
<td>16</td>
<td>142</td>
<td>126</td>
<td>89%</td>
<td>0.26 - 2.78</td>
<td>8.88</td>
</tr>
<tr>
<td>October 2007</td>
<td>10</td>
<td>119</td>
<td>49</td>
<td>41%</td>
<td>0.26 - 2.49</td>
<td>11.9</td>
</tr>
<tr>
<td>Totals</td>
<td>113</td>
<td>1132</td>
<td>770</td>
<td>68%</td>
<td>0.17 - 4.69</td>
<td>10.04</td>
</tr>
</tbody>
</table>

Every second month 2 random *E. coli* isolates were selected for reproducibility testing of the RAPD method. The isolates were subjected to a second round of genomic DNA isolation and RAPD analysis. Results of the second round were subsequently compared to the first round’s results. Reproducibility was tested on a total of 38 isolates, from which the highest deviation recorded were only 2 random loci. The majority of isolates rendered ≥90% similarities. Lanes 3 and 4 in Figure 4.7, D is an example of such a reproducibility test.
Figure 4.7: Examples of ethidium bromide stained 1.8% (w/v) agarose gels illustrating the banding patterns generated by the RAPD reactions. The first and the last lanes of each gel contain the molecular markers and the other lanes contain the RAPD reactions. The *E. coli* isolate numbers of each lane are presented in Appendix C. D) Lanes 3 and 4 is an example of a reproducibility test.
Figure 4.8: The phylogenetic tree constructed from RAPD data of the first 10 isolates.

Due to limitations of the phylogenetic tree construction software (TFPGA), 100 *E. coli* isolates have been selected that represented the entire study period and included all the vaccine isolates. These isolates were used to construct the final phylogenetic and antibiotic variance trees of the study and are illustrated in Figures 4.9 and 4.10 respectively. The isolate numbers designated to the *E. coli* isolates illustrated in the phylogenetic and antibiotic resistance variance trees and the months in which they were recovered are depicted in Table 4.3. The phylogenetic trees that include all 113 isolates on which RAPD analysis was done are presented in Appendix B.

The distance scale, spanning the top of the phylogenetic and antibiotic variance trees in Figures 4.8, 4.9 and 4.10, indicates the calculated distances between the isolates as fractions of 1 (e.g. 0.100, 0.200). These fractions represent the percentages by which the isolates differ from each other (% of genetic distance), therefore 0.100 equals 10% distance, 0.200 equals 20% distance, etc.
Table 4.3: The isolate numbers included in the phylogenetic and antibiotic resistance variance trees of *E. coli* pure cultures isolated during the sampling period.

<table>
<thead>
<tr>
<th>Month</th>
<th><em>E. coli</em> Isolate Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>September 2004</td>
<td>1</td>
</tr>
<tr>
<td>October 2004</td>
<td>2, 3, 4, 5, 6, 7, 8, 9, 10</td>
</tr>
<tr>
<td>May 2005</td>
<td>11, 12, 13, 14, 15</td>
</tr>
<tr>
<td>July 2005</td>
<td>16</td>
</tr>
<tr>
<td>October 2005</td>
<td>17, 18</td>
</tr>
<tr>
<td>November 2005</td>
<td>19, 20, 21, 22, 23, 24, 25</td>
</tr>
<tr>
<td>January 2006</td>
<td>26, 27, 28, 29</td>
</tr>
<tr>
<td>February 2006</td>
<td>30, 31, 32, 33</td>
</tr>
<tr>
<td>May 2006</td>
<td>34, 35, 36, 37, 38, 39, 40, 41</td>
</tr>
<tr>
<td>June 2006</td>
<td>42, 43, 44</td>
</tr>
<tr>
<td>July 2006</td>
<td>45, 46</td>
</tr>
<tr>
<td>August 2006</td>
<td>47, 48, 49, 50, 51, 52</td>
</tr>
<tr>
<td>September 2006</td>
<td>53, 54, 55</td>
</tr>
<tr>
<td>October 2006</td>
<td>56, 57, 58, 59, 60, 61, 62</td>
</tr>
<tr>
<td>November 2006</td>
<td>63, 64, 65, 66, 67, 68, 69, 70, 71, 72</td>
</tr>
<tr>
<td>December 2006</td>
<td>73, 74</td>
</tr>
<tr>
<td>January 2007</td>
<td>75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88</td>
</tr>
<tr>
<td>March 2007</td>
<td>89, 90</td>
</tr>
<tr>
<td>October 2007</td>
<td>91, 92, 93, 94, 95, 96, 97, 98, 99, 100</td>
</tr>
</tbody>
</table>
Figure 4.9: The phylogenetic tree of the 100 selected *E. coli* isolates constructed by using the RAPD analysis data. The vaccine isolates are also indicated.
The phylogenetic tree constructed from the RAPD data depicted in Figure 4.9 illustrates that, in general, the *E. coli* isolates originating from the same month were closer related to each other than to isolates from different months. These isolates were therefore grouped together in one or more clusters each consisting out of two or more isolates. There were exceptions to this pattern. Some individual isolates were substantially distant from all other isolates, and therefore not grouped into any clusters. It was also found that some isolates from different time periods were grouped into one cluster. The latter two occurrences were, however, less frequent than the former. A good example is the ten isolates from November 2006. Three isolates (64, 70 and 72) were highly distant from all other isolates. Two isolates (63 and 66) were highly distant from the rest of the month’s isolates but closely related to older isolates. Five isolates were closely related to each other and grouped into two clusters (65, 67, 71 in one cluster and 68, 69 in a second cluster).

Isolates from each month showing a genetic distance of 10% or less amongst each other could indicate that those isolates were actually only one *E. coli* strain that occurred in different milk samples. New *E. coli* isolates showing a genetic distance of 15% or less in comparison to isolates from previous months could be an indication of the re-occurrence of previous isolates (Figure 4.9). Most clusters of closely related isolates consist of small numbers of isolates which are mainly from the same month, except for one cluster in the top of the phylogenetic tree. It consists of 23 isolates all with distances of less than 5% amongst each other. These isolates also occurred in various months during the sampling period ranging from October 2004 to October 2007. This could be a strong indication of one *E. coli* strain which had a high re-emergence frequency within the herd, possibly from an unknown environmental source to which the herd was continuously exposed to.

One phenomenon that became apparent during this study was that the genetic distances between the *E. coli* isolates did not remain constant (except for the genetic distances of 0%) with the addition and integration of new isolates over time. This occurrence is due to the fact that RAPD analysis does not generate a known fixed number of predetermined loci. Scoring is done only by the presence or absence of bands at the generated loci. Appearance of new DNA fragments (from the newly added isolates), with sizes differing from all existing fragments, resulted in an increase in the number of loci generated. This in turn, increased the number of loci in which the
previous isolates are identical, resulting in a decrease in the genetic distance between those isolates.

This change in genetic distances between isolates is clearly illustrated when comparing the ten isolates in Figure 4.8 to the first ten isolates (isolate numbers 1 to 10) in Figure 4.9. The ten isolates in Figure 4.8 are the exact same isolates than the first ten in Figure 4.9, but they were analysed on their own, excluding the rest of the 90 isolates. The selected 100 isolates that were analysed together generated a total of 35 loci. On the contrary, the first ten isolates that were analysed on their own generated only 16 loci. Therefore, a higher number of generated loci would result in lower distances between existing isolates. For example, isolates 1 and 2 differed at three loci. Their direct distance using the raw data of Figure 4.9 is calculated by 3 loci of difference divided by 35 loci in total, as opposed to the raw data of Figure 4.8 where it is divided by the total loci of 16. Therefore, in Figure 4.8 their distance is illustrated as approximately 25% and in Figure 4.9 as only 9%. This trend, however, did not seem to alter the general genotype clustering of the isolates.
Figure 4.10: The antibiotic variance tree of the 100 selected *E. coli* isolates constructed by using the antibiogram data. The vaccine isolates are also indicated.
An antibiogram variance tree was constructed from the same 100 isolates that were selected for the phylogenetic tree. The antibiogram variance tree is depicted in Figure 4.10. As with the phylogenetic variance, the *E. coli* isolates have also shown great variance in their antibiotic resistance profiles. However, the variance profiles of the isolates between the two trees differ quite considerably.

The antibiotic resistance variance between isolates recovered in the same time frames (monthly) was high and many isolates recovered from different months and years were closely related to each other. There was a large amount of small clusters consisting of isolates that differed by 1 locus only (9% distance), but their distribution on the tree have shown no epidemiological significance. Only the isolates from October 2004 had greater similarities in their resistance profiles and were grouped into one cluster. The rest of the isolates were mainly scattered throughout the tree bearing no correlation to their isolation times. The antibiotic variance tree further demonstrated that there were no trends of resistance developing over time.

4.3.1 Comparison between the phylogenetic and antibiogram variance trees

Both the phylogenetic and antibiotic variance trees (Figures 4.9 & 4.10) displayed high levels of polymorphisms between the 100 isolates. This is, however, the only similarity between the trees. The distribution and clustering patterns of the isolates were greatly different between the two trees. As mentioned earlier, the antibiotic variance tree displayed a more irregular and random distribution of isolates. There were few clusters of isolates that displayed epidemiological significance and higher levels of similarities between isolates from different time periods were observed. The distribution of isolates across the phylogenetic tree was far less erratic. There were more clusters of isolates that originated from the same time period and fewer isolates showed close relatedness to isolates from different time periods. The clusters of similar time frames together with the variances between the isolates from different time periods did illustrate a high degree of epidemiological relevance.
4.4 Autogenous vaccine formulation and application

During the 41 month sampling period, 7 different autogenous vaccines were formulated and produced. Each vaccine consisted of a unique combination of *E. coli* and, in some cases, other mastitigen isolates. The 7 vaccines that were formulated and the months in which they were administered to the herd are depicted in Table 4.4.

Table 4.4: Types of autogenous vaccines produced with their *E. coli* isolate composition and the months the vaccines were administered to the dairy herd.

<table>
<thead>
<tr>
<th>Vaccine Type</th>
<th><em>E. coli</em> Isolates in Vaccine</th>
<th>Vaccination Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>R Vaccine 2</td>
<td>1, 17, 18, 24</td>
<td>January 2006, April 2006, July 2006</td>
</tr>
<tr>
<td>A Vaccine</td>
<td>45, 47, 48, 49</td>
<td>August 2006, October 2006</td>
</tr>
<tr>
<td>R Vaccine 3</td>
<td>57, 58, 59, 61, 63, 66, 69, 71</td>
<td>January 2007, March 2007</td>
</tr>
<tr>
<td>NR Vaccine</td>
<td>n/a</td>
<td>April 2007, May 2007</td>
</tr>
<tr>
<td>R Vaccine 4</td>
<td>84, 87</td>
<td>June 2007, July 2007, September 2007</td>
</tr>
<tr>
<td>R Vaccine 5</td>
<td>91, 93, 94, 96, 98, 99</td>
<td>November 2007, December 2007</td>
</tr>
</tbody>
</table>

Of the 7 vaccines that were formulated, 5 vaccines contained *E. coli* isolates that were selected using the RAPD data (designated vaccines R1 to R5) and one vaccine contained *E. coli* isolates selected from the antibiotic resistance variance data (designated vaccine A). In addition, one vaccine was formulated which did not contain any *E. coli* isolates (designated vaccine NR) and consisted only of 2 *Streptococcus dysgalactiae* and 1 *Streptococcus uberis* isolates. Vaccines R1, R3 and R5 contained only the listed *E. coli* isolates. Vaccines R2 and A each contained, in addition, two *S. dysgalactiae* isolates. Vaccine R4 contained two *S. dysgalactiae* and two *S. uberis*
isolates in addition to the two *E. coli* isolates. Figures 4.1 and 4.4 illustrate that the numbers of mastitis cases and *E. coli* isolates recovered during the vaccination period were significantly lower (P=0.0025 and P=0.0023 respectively) than the numbers recorded outside the vaccination period.

Vaccine R1 was formulated using the RAPD data of the isolates from September 2004, October 2004 and May 2005 (Table 4.3) and was administered to the herd within the first week of June 2005. The first vaccination appeared to be highly effective with the number of isolates dropping from 11 in May 2005 to only 4 in June 2005 (Figure 4.4). Vaccine R1 was administered to the herd again in July 2005 with the number of isolates still decreasing to 2 for the month and fell to 0 in the month of August 2005. September 2005 yielded only 1 isolate (Figure 4.4). The phylogenetic tree in Figure 4.9 illustrates that although October 2004 yielded more isolates than May 2005, the variance amongst May’s isolates were greater than October’s isolates. Isolates 12 and 13 from May 2005 were closely related to isolates 7, 8 and 10 from October 2004. This indicates that those isolates were causing persistent mastitis in the herd during that period. Therefore, the isolates selected for vaccine R1 represented most of the clusters of that time period, which could account for its initial effectiveness.

Vaccine R1 administration was continued in the months October, November and December 2005. However, the rise in numbers of *E. coli* isolates continued in November 2005 and despite the decrease in December, the number of *E. coli* isolates was still higher than the 3 preceding months with an increase again in January 2006 (Figure 4.4). There was also an upward trend in the numbers of mastitis cases within the herd during this period (Figure 4.1). The RAPD analysis revealed that the majority of isolates from October 2005, November 2005 and January 2006 were highly distant from the isolates in vaccine R1. This accentuated the need to formulate a new vaccine consisting of new isolates.

Vaccine R2 was formulated, which included isolates from September 2004, October 2005 and November 2005 (Table 4.3), and was administered to the herd in January, April and July 2006. Despite a few sporadic decreases in the number of *E. coli* isolates during the application period of vaccine R2, there was still an overall increase in the number of *E. coli* isolates (Figure 4.4). A highly similar pattern was observed in
the numbers of mastitis cases (Figure 4.1). This indicated that although the *E. coli* mastitis incidences were not alarmingly high, vaccine R2 did not seem to be as effective as vaccine R1. The RAPD analysis revealed again that the isolates occurring from January to July 2006 were highly distant from the isolates in vaccine R2 as well as the isolates from 2005. This could be an indication of a stronger challenge of new emerging *E. coli* strains within the herd. However, due to the unavailability of RAPD analysis during this time period this data was only revealed at a later stage. At that specific time an alternative analysis method had to be utilised in order to formulate a vaccine from the isolates occurring during that period (January to July 2006).

Antibiotic resistance variance was the method used to formulate vaccine A. This vaccine contained one isolate from July 2006 and three isolates from August 2006. Due to an increase in *S. dysgalactiae* isolates from June to August 2006, two of those isolates were included in this vaccine (Figure 4.2). Vaccine A was administered to the herd in August and October 2006. There was an initial decrease of *E. coli* isolates in September 2006, but from October 2006 (after the second vaccination) to January 2007 there was a vast increase in the number of isolates reaching levels as high as 12 and 14 in November 2006 and January 2007 respectively. This was by a great margin the highest number of isolates recovered since the first vaccination in June 2005. Based on these findings it was clear that vaccine A was a highly ineffective.

The reason for the ineffectiveness of vaccine A becomes evident when comparing the RAPD analysis and antibiotic variance data sets (illustrated in Figures 4.9 and 4.10) of the *E. coli* isolates in vaccine A. According to the antibiotic variance tree (Figure 4.10), the four isolates were highly distant from each other and for this reason they were selected for the vaccine. When RAPD analysis was resumed in mid December 2006, the data revealed that all the *E. coli* isolates from August 2006 were in fact very closely related and therefore potentially represented a single strain (Figure 4.9). Figure 4.9 further illustrates that the isolates from August 2006 are highly distant from all the other isolates in the study. This gives an indication that because three of those isolates were in vaccine A, the protection the vaccine rendered against that specific strain was highly effective. The strain was therefore completely eliminated from the herd and never reoccurred. However, because vaccine A did not contain strains of sufficient variation, this seemed to render the herd more susceptible to other *E. coli* strains.
The RAPD analysis data revealed (at a later stage) that all three isolates from September 2006 were identical. These isolates were also very closely related to 5 isolates from November 2006, 1 isolate from December 2006 and 5 isolates from January 2007. In addition, a few isolates from October 2006, November 2006 and January 2007 were closely related to each other and were highly distant from the isolates in vaccine A (Figure 4.9). This clearly indicates that there was a high reoccurrence rate of isolates during the period of vaccine A application. This would have been earlier detected and prevented if RAPD analysis was done during this period. The isolates from May 2006 were the only group that have shown great phylogenetic variance amongst each other and were distant from all other isolates, which indicates a low reoccurrence rate of these strains. The reason for this incidence is unknown and could be attributed to varying environmental, climate or herd management conditions. In contrast to these findings, the antibiotic variance tree revealed only high levels of variance between the isolates of this time period with no indication of reoccurrence and epidemiological significance.

Vaccine R3 was formulated, using RAPD analysis that was resumed in mid December 2006, and was administered to the herd in the beginning and end of January 2007 and again at the beginning of March 2007. Due to the increasing occurrence of *E. coli* mastitis during the vaccine A application period, vaccine R3 consisted of *E. coli* isolates only and contained 8 isolates. Vaccine R3 contained 4 isolates from October 2006 and 4 isolates from November 2006. This vaccine proved to be highly effective. No *E. coli* were isolated during February 2007 and only 4 were isolated in March 2007. This occurred despite the fact that vaccine R3 did not contain any isolates from December 2006 and January 2007. RAPD analysis revealed that the isolates in vaccine R3 were very closely related to the vast majority of the isolates from December 2006 and January 2007. This demonstrated the effectiveness of the RAPD formulated vaccine.

Vaccine NR was formulated (following the R3 vaccinations) and did not contain any *E. coli* isolates. It was administered to the herd in April 2007 and May 2007. The reason for excluding *E. coli* from the vaccine was the drastic reduction in *E. coli* mastitis cases and an emergence of *S. uberis* and *S. dysgalactiae* mastitis cases during February and March 2007 (Figure 4.2). For this reason only those two organisms were included in vaccine NR.
During May 2007 the number of *E. coli* isolates started to increase. However, no RAPD analysis was done on these isolates. Vaccine R4 was formulated which contained 2 isolates from January 2007. The vaccine was administered to herd in June, July and September 2007. Although the effectiveness of the vaccine could not be completely determined due to the fact that no sampling was done on the farm from June to August 2007, the total number of mastitis cases within the herd remained very low during this period (Figure 4.1). In September 2007 when sampling was resumed, only 3 *E. coli* isolates were isolated from 30 milk samples (Figure 4.4). This could be an indication that vaccine R4 was effective, despite the fact that RAPD analysis was not done on those 3 isolates. However, in October 2007 10 *E. coli* isolates were recovered from the samples, all being highly distant from the two isolates in vaccine R4. Variance amongst the 10 isolates themselves was also high.

Vaccine R5 was formulated containing 6 *E. coli* isolates from October 2007 and was administered to the herd in November and December 2007. These vaccinations resulted in the reduction of the numbers of isolates to 5 in November and 0 in December 2007 (Figure 4.4). Although RAPD analysis was not done on the 5 isolates from November 2007, the rapid reduction in *E. coli* mastitis cases to 0 in December 2007 has proven this vaccine to be highly effective.

The vaccination in December 2007 was the final vaccination given to the herd. From January 2008, the herd and mastitis management was continued without the application of DCA vaccines. Sampling and pathogen isolation was also discontinued. During January and February 2008 mastitis cases were still very low. This was expected since protection is expected to last for approximately 3 months after vaccination. Protection is depended on the non-occurrence of new strains. From March 2008 and onwards the number of mastitis cases started to increase drastically, reaching numbers as high as approximately 50 in May and June, 108 in November and 72 in December of 2008. The pathogens responsible for these high mastitis incidences were not identified. Despite this, the data still proves that the DCA vaccines were an exceptionally efficient tool in controlling *E. coli* mastitis within the herd.
5.1 Introduction

Bovine mastitis is a highly complex disease with many different species of bacteria being able to cause mastitis. Various host (cow), environmental and pathogen characteristics all play an interacting role in the susceptibility of a cow and the severity of mastitis (Lehtolainen et al., 2003). *E. coli*, however, is becoming an increasing mastitis risk to dairy herds due to high genetic variability between the vast number of strains and the fact that it is an environmental pathogen that can be contracted from various different sources (Suojala et al., 2011). Antibiotic treatment regimes, mastitis vaccines and proper cleaning and hygiene practices all fail to give consistent effective solutions for the control of *E. coli* mastitis (Denis et al., 2009; Hurley, 2010). In the current study, a specific monitoring and vaccine formulation strategy was adopted to control *E. coli* mastitis in a dairy herd. The strategy mainly involved the combination of antibiotic variance data, a reliable molecular typing method and the formulation of a farm specific vaccine that gives protection in the udders of the cows.

5.2 Mastitis occurrence, samples and pathogen isolates

The number of mastitis cases within dairy herds usually tends to be higher during the wet and rainy season than in the dry season (Rahman et al., 2009). This phenomenon is related to the fact that warm and wet weather conditions favour the growth of bacteria in the environment (Kusiluka and Kambarage, 1996). The Darling area in the Western Cape Province falls in a winter rainfall region (Neuhoff, 2010) which translates to cold and wet conditions during winter. Cold conditions, on the other hand, can lead to chapped and cracked teats, which increases the ability of bacteria to colonize in the teats and cause mastitis (Hurley, 2010).

The cows were mainly housed on soil during the study period. This could have lead to muddy conditions contaminated with faeces in the rainy season. These conditions coupled to the factors mentioned above could explain the fact that, historically,
mastitis cases on this farm were usually higher during the rainy season than during the dry season. This muddy and faecal contaminated environment to which the cows were exposed to could also be the reason why *E. coli* was found to be the main cause of mastitis within the herd. This occurrence can be attributed to the fact that *E. coli* is shed in faeces into the environment (Van Kessel *et al.*, 2004).

Figure 4.1 illustrates that during the entire vaccination period the monthly numbers of mastitis cases were consistently very low, irrespective of the seasonal changes. The months of May and August 2007, which are both wet months, yielded two out of the three lowest monthly number of mastitis cases during the study period. This trend gives a strong indication that seasonal variance did not have an influence on the mastitis data of the herd during the vaccination period.

There was a general association between the numbers of mastitis cases and *E. coli* isolates recovered during the study period. The only exception was in June, July and August of 2005 (Figures 4.1 and 4.2). The exact reason for this occurrence is unknown. Previous studies have shown that toxins are released upon cell death of *E. coli* (Green, 1998; Petersson-Wolfe and Currin, 2011). Zhao and Lacasse (2008) demonstrated that that the host immune response as well as bacterial toxins can lead to mammary tissue damage. This tissue damage in turn leads to extracellular fluids to enter the glands and mix with the milk, which cause the formation of watery milk with flocculates. The initial high numbers of *E. coli* mastitis cases present in the herd prior to the vaccination period could have lead to elevated levels of immune response and toxin release in the cows during the first two vaccinations. This in turn could have accounted for higher numbers of positive mastitis tests even in the absence of potential culturable mastitigens. Figure 4.5 further illustrates that the average percentage of mastitigens isolated from the samples during this period was very low. Due to the complex nature of mastitis, it has been found that there is not always a direct correlation between positive on-site mastitis tests and bacterial culture tests (Schipper *et al*., 1974; Hurley, 2010).

### 5.3 Antibiotic resistance amongst *E. coli* isolates

The use of antibiotics for the treatment of cows with clinical mastitis is considered an important method to combat mastitis in dairy herds (Suojala *et al*., 2010). Antibiotic
resistance profiles of the *E. coli* isolates in this study (Figure 4.6) demonstrated that, in general, a large percentage of isolates were resistant to the majority of antibiotics tested. However, most were susceptible to Cefuroxime 2 and Polymyxin B. The reason for this can be attributed to the fact that none of these 2 antibiotics were used in the herd.

The antibiogram variance tree in Figure 4.10 illustrates that, in general, no relation was found between the time frames from which isolates were recovered and their antibiotic resistance relatedness. This irregular occurrence of antibiotic resistance could be attributed to the fact that soil organisms can serve as a reservoir for the transfer of antibiotic resistance (Bester and Essack, 2010). The constant mixing of faecal contamination into the soil within the cows’ environment could have brought the shed *E. coli* and soil bacteria into close proximities to each other, resulting in the constant exchange of antibiotic resistance genes. These *E. coli* can in turn infect the udders through the teat canals as the cows move around and lay down on the ground (Jones and Swisher, 2009; Blowey and Edmondson, 2010).

Despite this irregular occurrence of resistance to most of the antibiotics, the high occurrence of the CLNN resistance pattern within the isolates indicated the persistence of these antibiotic resistance genes within the *E. coli* population on the farm. Since Amoxicillin (similar to Ampicillin) was the main antibiotic used to treat severe mastitis cases on the farm (before, during and after the study), it was expected that resistance to this antibiotic would be a major feature in this population. However, the resistance, intermediate and sensitive profiles of the isolates to this antibiotic were highly erratic throughout the study period. A study of faecal *E. coli* in dairy calves found the persistent occurrence of multi-resistant isolates in the herd, even in the absence of antibiotic treatment (Berge *et al.*, 2005). A study in Canada has also found that previous treatments with antimicrobials within cattle herds did not seem to have an effect on the occurrence of antibiotic resistance genes among the *E. coli* isolates (Gow *et al.*, 2008). This could potentially explain the irregular occurrence of Amoxicillin resistance within the *E. coli* population in this study, despite the regular use of Amoxicillin in this dairy herd. What was observed is that 100% of the isolates were resistant to Cloxacillin, a semi-synthetic beta-lactam antibiotic.
Nearly all *E. coli* isolates (99%) were resistant to Lincomycin. This was expected. It is known that *E. coli* strains are normally resistant to this antibiotic (Spízek and Rezanka, 2004; Skoufos *et al.*, 2006). Lincomycin was included in the antibiogram tests since it forms part of the standard test kit that were used to analyse all pathogens isolated in this study.

The present study illustrated highly irregular occurrences of antibiotic resistance as well as persistent resistance patterns amongst the isolates. This accentuates the need for constant antibiotic resistance monitoring of mastitis causing *E. coli* in order to employ potentially more efficient antibiotic treatment regimes. However, there is a continuous risk of inducing additional resistance amongst the current bacterial population (Santos *et al.*, 2010). As an alternative, preventative control measures include the use of teat dips, germicides in cleaning solutions and the provision of dry housing conditions for the cows. The drawbacks of these measures are that environmental organisms can be resistant to germicides and teat dips (Hurley, 2010).

### 5.4 RAPD Development

One of the most important requirements of the RAPD analysis method is a high degree of reproducibility. Two published RAPD methods for *E. coli* (Radu *et al.*, 2001; Fei *et al.*, 2003) were individually tested and with both methods the reproducibility was unsatisfactory. In some cases the distances after retesting of the same isolate were as high as 50%. Only Radu *et al.* (2001) mentioned that their method generated reproducible patterns, where Fei *et al.* (2003) did not make mention reproducibility testing. However, the stigma of low inter-laboratory reproducibility of the RAPD method (Mienie, 2003) could account for the low reproducibility found with these published methods when tested in the current study.

The low levels of reproducibility of the two published methods led to the need to develop an in-house RAPD method for *E. coli*. This method gave reproducible profiles. The aim, when developing the in-house method, was to design a 10-mer primer with an annealing temperature higher than 40°C. The optimum annealing temperature of the selected primer was 42°C. Most of the published primers have annealing temperatures of below 40°C (Radu *et al.*, 2001; Fei *et al.*, 2003; Lin and Lin, 2007;
Kiliç et al., 2009). It is believed that the higher annealing temperature of the in-house developed RAPD primer reduced the incidence of mismatch priming on the template and therefore increases the reproducibility and stability of the method (Dieffenbach et al., 1993).

5.5 The phylogenetic and antibiogram variance trees

The RAPD generated polymorphisms of the *E. coli* isolates ranged from highly distant to identical banding patterns (Figure 4.9; Table 4.2). Several other studies have found that RAPD analysis generated sufficient levels of polymorphisms for epidemiological analysis even if different RAPD primers were utilised in the various studies (Watt et al., 2003; Lin and Lin, 2007; Kiliç et al., 2009).

The phylogenetic tree in this study, based on the RAPD data (Figure 4.9), presented a holistic view of the *E. coli* isolates from which epidemiological trends were exhibited. These trends were of great value in *E. coli* mastitis monitoring during the study period. Genetic variation from previously occurring isolates as well as genetic diversity amongst presently occurring isolates were readily detected. A study on the prevalence of *E. coli* O157 in feedlot cattle was also able to present the endemic character of the isolates using RAPD analysis (Vidovic and Korber, 2006). Epidemiological trends presented by the RAPD method could also aid in indicating whether *E. coli* contamination originated from single or numerous sources, which can result in the selection of more efficient control measures (Vidovic and Korber, 2006). Radu et al. (2001) also suggested that molecular typing methods can be very useful in identifying critical control points in the food industry for the control of *E. coli* O157:H7.

The antibiotic variance tree (Figure 4.10) presented a high degree of differences amongst the *E. coli* isolates. The tree was unable to exhibit any epidemiological trends of value. The only trend observed was a highly erratic occurrence of antibiotic resistance profiles over time. This can be attributed to the fact that antibiotic resistance profiles of bacteria are more related to environmental factors to which they are exposed and less related to their inherent genetic composition (Hoyle et al., 2006). The antibiotic variance data generated numerous pairings of isolates that differed only at one random locus. These pairings appeared to be of no epidemiological value. This phenomenon was overcome by Moneoang and Bezuidenhout (2009) that utilised the
exact diameter data of the inhibition zones to determine variances. The use of
diameter measurements increases the resolution and discriminative power of
antibiotic variance trees. However, antibiotic variance can only illustrate the movement
of antibiotic resistance genes within the environment, with no relation to the
epidemiological spread of the bacteria themselves (Khan et al., 2002; Santos et al.,
2010).

5.5.1 Comparison between the phylogenetic and antibiogram variance
trees

No similarities were found in the clustering patterns of the isolates between the
phylogenetic and antibiotic variance trees. This high level of differences between the
two trees was expected. The reason for this phenomenon lies in the difference
between the genetic elements that each method targets. Antibiotic resistance of
bacteria are mainly located on less stable genetic elements such as plasmids and
transposons (Berge et al., 2005). These elements can be obtained from the
environment, even if they originated from different species of bacteria (Bester and
Essack, 2010). Antibiotic resistance can also be obtained at any point in time
regardless of the evolutionary stage of a population or antibiotic usage at the specific
location (Berge et al., 2005). It is most probably due to these factors that gave rise to
the erratic distribution of isolates in the antibiotic variance tree.

The RAPD analysis method targets the genome of an organism (Radu et al., 2001; Lin
and Lin, 2007). The genome is a highly stable genetic element that mostly contains
genetic elements which are transferred vertically from one generation to the next
(Trun and Trempy, 2004). Although RAPD primers do not target specific
predetermined loci, there is a smaller chance that unstable elements would be
amplified. For these reasons, the phylogenetic tree produced genomic variance
profiles which appeared to be of great epidemiological value. Therefore sequence
analysis of the RAPD generated bands could give further insight into the
epidemiological and pathogenic nature of mastitis causing E. coli.
5.6 Vaccine formulation, application and *E. coli* monitoring

The autogenous vaccine formulation strategy adopted by DCA is to produce mastitis vaccines by using the pathogens isolated on a specific farm and apply the vaccine on that farm only. There are two major factors influencing autogenous vaccine formulation: 1) Limitations in the number different isolates that can be included in one vaccine and 2) the vaccine isolates must be homologous to the field strains causing the disease in question (Funk *et al.*, 2009; Cooper, 2010a). Several studies have revealed that high genetic variation at specific antigenic loci can reduce vaccine efficacy (Russo *et al.*, 2007; Feavers and Pizza, 2009; Jongwutiwes *et al.*, 2010). Antibodies that are produced by using a specific antigen do not necessarily cross-react with variants of that antigen. Therefore protection against the variants could be inadequate (Russo *et al.*, 2007). It has also been found that animals do not react equally well to all antigens that they are exposed to. It would therefore be advantageous to limit the number of antigens during vaccine formulation (Talbot and Lacasse, 2005; Denis *et al.*, 2009).

The factors mentioned above together with the large number of *E. coli* isolates initially recovered from the herd at the start of this study, accentuated the necessity for a vaccine strain selection method. RAPD analysis was the main screening method selected to serve this purpose.

The main trend arising from the vaccination, *E. coli* monitoring and RAPD analysis data was that different vaccines containing new prototypic isolates had to be continuously formulated in order to effectively control *E. coli* mastitis in the herd. Not one single vaccine was able to provide prolonged protection against new *E. coli* infections. Similar results were found with the *E. coli* J5 vaccines where the researchers have also shown its inability to protect animals against new infections (Talbot and Lacasse, 2005; Wilson *et al.*, 2009). Autogenous vaccines in general have been found to lack prolonged protection against the diseases they were intended for, mainly because of antigenic variance arising amongst the pathogens (Denis *et al.*, 2009; Meulemans *et al.*, 2011). This trend accentuates the importance of the close relation required between field strains and vaccine strains.
Results from the present study demonstrated that RAPD data were more effective for vaccine formulation than antibiotic variance data. This gives an indication that this specific RAPD analysis method is able to illustrate antigenic variances between isolates in addition to purely genomic variances. A similar relation between RAPD data and antigenic properties was found in a study which tested the potential of an O-antigen deficient *E. coli* strain for vaccine purposes (Russo *et al.*, 2007). These authors (Russo *et al.*, 2007) found that antibodies produced from this *E. coli* strain cross-reacted far more strongly with closely related RAPD generated genotypes than distantly related ones. The results from the present study further demonstrated that RAPD based formulation of inactivated polyvalent *E. coli* vaccines, together with regular strain updating of the vaccines, proved to be highly effective in the prolonged control of *E. coli* mastitis in the herd (control was exhibited for the entire duration of the vaccination period). A study of *Staphylococcus aureus* mastitis also revealed that accurate genotyping of strains can greatly increase the effectiveness of strain-specific cures (Barkema *et al.*, 2006). All of these findings accentuate the need to further investigate the direct relation between the RAPD generated strains and their immunological properties.

RAPD analysis enabled the selection of prototypic vaccine strains which seemed to cover the entire spectrum of antigenic variance occurring within the herd. Genetic variation from vaccine strains over time was also readily detected by RAPD analysis, allowing for immediate updating of vaccines. This prevented major increases in *E. coli* mastitis cases during the reformulation periods and resulted in decreases in the numbers of cases after application of the new vaccines.

The data presented in this study strongly indicated that accurate genotyping, accurate polyvalent vaccine formulation, constant *E. coli* monitoring and correct timing of vaccine application all play an essential part in the successful control of *E. coli* mastitis. This methodology could be extrapolated to other mastitis causing species of bacteria in order to provide a wider range of effective control measures directed against general mastitis.
CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The aim of this study was to determine the genetic and antibiotic resistance variability amongst mastitis causing E. coli and to establish whether these data sets could be applied to formulate autogenous vaccines for the effective control of E. coli mastitis.

Five objectives were formulated to achieve the aim of this study. The objectives were set out to achieve three main goals: to establish a reliable genotyping method of E. coli, to determine the epidemiological value between genotyping and antibiotic variance profiles for vaccine formulation and to monitor the effectiveness of the applied vaccines.

(i) The development of a fast, reliable and reproducible genotyping method for E. coli.

The two published RAPD analysis methods that were tested gave highly unsatisfactory results regarding reproducibility. The in-house method that was developed yielded high levels of reproducibility and polymorphisms. The method was also able to provide an insightful illustration of the epidemiological occurrence of the E. coli isolates during the study period.

(ii) The antibiotic resistance profiles of the E. coli isolates.

The E. coli isolates displayed high levels of resistance to most of the antibiotics. Nearly all the isolates were multidrug resistant (with the exception of one isolate) bearing resistance to at least three antibiotics. There were no trends arising in the resistance profiles of the isolates and the occurrence of various resistant and sensitive traits over time were highly erratic. This data showed that antibiotic resistance have spread vastly within the herd's environment at earlier stages and that highly resistant E. coli isolates were persistently present in the herd. For these reasons antibiotic
treatment of mastitis causing *E. coli* in the present herd is considered to be ineffective and costly.

(iii) **The correlation between the antibiotic variance and phylogenetic profiles of the *E. coli* isolates.**

There were no similarities found between RAPD generated variance and the antibiogram variance profiles of the isolates. The antibiotic variance tree has illustrated great variance between all the isolates with no relation to the isolation time periods of the isolates. There were also no relevant relations found between the vaccine isolates and new occurring isolates during the vaccination period. For these reasons antibiotic variance profiles are considered to be highly unreliable for epidemiological studies of mastitis causing *E. coli*. RAPD analysis, on the other hand, created a meaningful holistic view of the genetic variance dynamics of *E. coli* isolates which occurred within the dairy herd. Strong relations were found between the isolation periods of the isolates and the formation of isolate clusters on the phylogenetic tree. The phylogenetic tree also provided insightful data between the vaccine strains and new occurring isolates during the vaccination period.

(iv) **Autogenous vaccine formulation, application and the monitoring of *E. coli* isolates within the vaccination period.**

RAPD analysis was a useful tool in the autogenous vaccine formulation process. During the study period, five vaccines were formulated using RAPD analysis and one vaccine was formulated using antibiotic variance analysis. These vaccines each contained a unique combination of newly occurring *E. coli* isolates and were administered nasally to the herd at different time periods. All vaccines formulated utilizing RAPD analysis were highly effective in reducing *E. coli* mastitis incidences. The vaccine formulated using antibiogram variance data was far less effective which resulted in an overall rise in the number of *E. coli* mastitis cases during its application period. The RAPD data was able to identify the reason for its ineffectiveness where the antibiogram variance data were unable to illustrate any meaningful explanation. Genetic variance from vaccine isolates were readily detected by RAPD analysis. This enabled the manufacturer to update the vaccines and apply the new vaccines to the
herd before a considerable increase in mastitis cases could occur. This specific RAPD method together with an efficient mastitigen monitoring program (e.g. frequent and proper sampling practices, efficient bacteriological analysis) are therefore considered to be the key in formulating and updating autogenous vaccines that are highly effective in controlling *E. coli* mastitis within dairy herds.

**6.2 Recommendations**

1) Inter-laboratory testing of this specific RAPD analysis method should be conducted in order to determine the robustness of the procedure.

2) Immunological cross reactions between closely related as well as distant *E. coli* isolates should be tested under controlled laboratory conditions. This will determine the degree of correlation between the RAPD generated variance and the antigenic variance of the isolates.

3) Specific common RAPD generated bands and uncommon bands must be identified to undergo gel extraction, cloning and sequencing. The sequence data together with the epidemiological data could provide valuable information regarding the pathogenesis of mastitis causing *E. coli*. It can also aid in the development of a new typing method targeting a fixed number of disease related loci. The sequence data could reveal specific common antigenic properties of *E. coli* that can be used to develop an effective general *E. coli* mastitis vaccine.

4) RAPD analysis can be conducted on *E. coli* isolates from different farms in the same region and from different regions. This could give further insight into the regional epidemiology of *E. coli* mastitis.

5) Mastitis pathogen monitoring in the herd should be resumed for at least three months in order to determine the current mastitis pathogen profile in the herd. The data would also indicate whether *E. coli* returned as the dominant cause of mastitis on the farm.
6) Future studies should include negative control groups which do not get any vaccinations and additional treatment regimes should be documented and restricted.

This study has demonstrated that the in-house developed RAPD analysis method of *E. coli* was a highly effective tool in autogenous vaccine formulation and these vaccines have successfully controlled *E. coli* mastitis within the herd. It was also demonstrated that the antibiotic variance profiles differed from the phylogenetic profiles and the phylogenetic profiles were able to illustrate valuable epidemiological information. Thus, the aim was achieved by the successful execution of the five objectives.
REFERENCES


80


81


## APPENDIX A

### Antibiotic resistance data

**Table A1**: The antibiogram data of the selected 100 isolates derived from the disk diffusion method.

<table>
<thead>
<tr>
<th>Isolate no</th>
<th>Ampicillin/Amoxicillin</th>
<th>Cefuroxime 2</th>
<th>Cloxacillin</th>
<th>Lincomycin</th>
<th>Neomycin</th>
<th>Novobiocin</th>
<th>Polymyxin B/Collistin</th>
<th>Streptomycin</th>
<th>Tetracycline</th>
<th>Cephalothin 1/Cephalexin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td>3</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>5</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>6</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>7</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>8</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>9</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>10</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>11</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>12</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>13</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>16</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td>17</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>18</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>21</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td>22</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>23</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>24</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td>25</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>26</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>27</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>28</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>29</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>30</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>31</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>33</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>34</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>37</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>41</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
</tr>
<tr>
<td>---</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
<td>-----------</td>
<td>--------------</td>
</tr>
<tr>
<td>44</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
</tr>
<tr>
<td>45</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>50</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>51</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>52</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>57</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>59</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>60</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>61</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>63</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>64</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>67</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>69</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>---</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>70</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>71</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>72</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>73</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>74</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>75</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>76</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>77</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>78</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>79</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>80</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>81</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>82</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>83</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>84</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>85</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>86</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>87</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
</tr>
<tr>
<td>88</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>89</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>90</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>91</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>92</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Intermediate</td>
</tr>
<tr>
<td>93</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Resistant</td>
</tr>
<tr>
<td>94</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
</tr>
<tr>
<td>95</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>---</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>96</td>
<td>Intermediate</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>97</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>99</td>
<td>Sensitive</td>
<td>Sensitive</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Sensitive</td>
</tr>
<tr>
<td>100</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Intermediate</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Resistant</td>
</tr>
</tbody>
</table>
APPENDIX B

RAPD generated phylogenetic trees of the 113 *E. coli* isolates

*Figure B1*: The RAPD generated phylogenetic tree of *E. coli* isolated from September 2004 to August 2006 (65 isolates).
Figure B2: The RAPD generated phylogenetic tree of *E. coli* isolated from September 2006 to October 2007 (48 isolates).
APPENDIX C

E. coli isolates that are presented in Figure 4.7

Table C1: Isolate numbers of the E. coli isolates’ RAPD profiles that are presented in the gel lanes of Figure 4.7 A to D.

<table>
<thead>
<tr>
<th>Lane 2</th>
<th>Figure 4.7 A</th>
<th>Figure 4.7 B</th>
<th>Figure 4.7 C</th>
<th>Figure 4.7 D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 3</td>
<td>Isolate 45</td>
<td>Isolate 79</td>
<td>Isolate 89</td>
<td>Isolate 95</td>
</tr>
<tr>
<td>Lane 4</td>
<td>Isolate 46</td>
<td>Isolate 80</td>
<td>Isolate 90</td>
<td>Isolate 96</td>
</tr>
<tr>
<td>Lane 5</td>
<td>Isolate 53</td>
<td>Isolate 81</td>
<td>NPOS**</td>
<td>Isolate 96</td>
</tr>
<tr>
<td>Lane 6</td>
<td>Isolate 54</td>
<td>Isolate 82</td>
<td>NPOS**</td>
<td>Isolate 98</td>
</tr>
<tr>
<td>Lane 7</td>
<td>Isolate 55</td>
<td>Isolate 83</td>
<td>NPOS**</td>
<td>Isolate 99</td>
</tr>
<tr>
<td>Lane 8</td>
<td>NP*</td>
<td>Isolate 85</td>
<td>NPOS**</td>
<td>Isolate 100</td>
</tr>
<tr>
<td>Lane 9</td>
<td>Isolate 86</td>
<td>NPOS**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lane 10</td>
<td>Isolate 87</td>
<td>NPOS**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lane 11</td>
<td>Isolate 73</td>
<td>NPOS**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lane 12</td>
<td></td>
<td>NPOS**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Isolates that were Not Presented in Figures 4.9 & 4.10.
** Isolates that were Not Part of this Study.