SURVEILLANCE OF POTENTIALLY HAZARDOUS BACTERIA IN MILK AND VEGETABLE PRODUCE: BACTERIAL CONTENT AND ANTIBIOTIC RESISTANCE

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DECLARATION OF INDEPENDENT WORK

I, HANLI DE BEER, do hereby declare that this research project submitted for the degree MAGISTER TECHNOLOGIAE: ENVIRONMENTAL HEALTH, is my own independent work that has not been submitted before to any institution by me or anyone else as part of any qualification.

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ABSTRACT

The community and employees are adversely affected through consumption and exposure to hazardous bacteria. Often cause-related illness can be of a transient nature with self-limiting effects that can be serious, or even life-threatening or fatal. The highest possible quality of milk should therefore reach the consumer. Not only microbiological counts, but the reliable detection of pathogens and antibiotic residue should also be part of routine analysis.

The microbiological status of milk samples from all categories collected in the Bloemfontein area during 1997 and 1998 were generally considered unsatisfactory. Some of the milk samples from individual cows tested in this study were of a good hygienic quality. However, as soon as milk lots were combined as a batch, the quality declined remarkably. The majority of the milk sold at depots did not conform to one or more of the standards as specified by law.

A total of 55 milk samples were collected. Of a total of 20 raw batch samples tested, 85% did not conform to the standards for raw milk, whilst 82.6% of 23 depot and industrially pasteurised milk samples did not conform. Of concern was the fact that 5/19 depot milk samples failed all three microbiological count standards.

In this study, apart from *Staphylococcus aureus, Listeria* species and two ß-haemolytic streptococci, no other pathogens were detected. The *Listeria* isolation method is well-developed and consists of two enrichment stages. Many potentially promising pathogens were eventually identified as opportunistic pathogens, such as *Enterobacter sakazakii* and *Ent. agglomerans*.

Polymerase chain reaction (PCR) is a very convenient method to be used for the identification of organisms and was used as alternative to conventional microbiological methods for pathogen detection. Milk, however, is not an easy medium to incorporate into the PCR system. Difficulty was experienced in

obtaining good reproducible target DNA, and the PCR product sizes were not always as expected. It will be necessary to test local strains and to continuously correlate and update PCR performance.

In this study it was found that certain factors in milk had an influence on the PCR results. Freezing and thawing of a milk sample adversely affected PCR results. The Dynabead® DNA concentration concept, which is vital for the detection of low cell numbers, performed very well, but was also affected by the freezing and thawing process. The conventional microbiological and PCR results concerning *L. monocytogenes* were in accordance. The use of PCR for the detection of *Campylobacter* and *Brucella* appeared promising. Although PCR technology can be fraught with inherent problems, it remains the most comprehensive and convenient option for future rapid detection/identification of foodborne pathogens.

Problems experienced with antibiotic administration for a variety of reasons have lead to the misuse of antibiotics by farmers. Lack of knowledge and the use of growth promoters could be reasons for the incidence of antibiotic-resistant strains in the environment. Gram-positive and gram-negative strains were selected from milk, vegetable and farm environmental samples and investigated for antibiotic-resistant genes. The resistance profiles of staphylococci isolated from raw and pasteurised milk during 1997 and 1998 showed *S. aureus* resistance to three or more antibiotics with streptomycin, tetracycline and cephalosporin resistance evident. The majority of antibiotic-resistant coagulase-negative staphylococci (CNS) showed resistance to ciprofloxacin, streptomycin and oxacillin.

Milk is one of the most nutritious products available at an affordable price. In a country where the majority of the community lives in poverty, it is a product of critical importance. Milk that is bought directly from the farmer or at depots is usually cheaper than those sold at shops. In this study it was clear that milks did not comply to microbiological regulations. Pathogens and antibiotic resistant strains were detected. Consumers have the right to receive milk of good quality, which is free from pathogens and antibiotic residue.

OPSOMMING

Die gemeenskap en werkers kan benadeel word deur die inname van of blootstelling aan siekteveroorsakende bakterieë. Siektetoestande kan van verbygaande aard wees, maar kan soms komplikasies en selfs die dood tot gevolg hê. Dit is dus noodsaaklik dat melk van 'n hoë kwaliteit die verbruiker bereik. Ontleding vir patogene mikro-organismes, asook betroubare antibiotika-residuetoetsing, behoort deel uit te maak van roetine-ontledings.

Die studie wat tydens 1997 en 1998 in die Bloemfontein-gebied gedoen is, het getoon dat die kwaliteit van melk uit verskeie kategorieë, nie aan die wetlike standaarde voldoen het nie. Melkmonsters wat van individuele koeie geneem is, was meestal van 'n goeie gehalte. Sodra die melk egter in 'n groot lot saamgevoeg is, het die gehalte aansienlik verswak. Die meerderheid van melkmonsters wat by depots verkoop was, het een of meer standaardtelling soos deur die wet vereis, oorskry.

'n Totaal van 55 melkmonsters was versamel. Van die 20 rou melkmonsters het 85% nie aan die gespesifiseerde standaarde vir rou melk voldoen nie, terwyl 82.6% van die 23 depot en industrieel gepasteuriseerde monsters, onder standaard was. Die feit dat 5/19 depotmonsters al drie mikrobiologiese standaarde oorskry het, was kommerwekkend.

Die enigste patogene organismes wat in hierdie studie geïsoleer was, was *Staphylococcus aureus, Listeria* spesies en twee ß-haemolitiese streptococci. Twee verrykingsstappe maak die isolering van *Listeria* spesies redelik suksesvol. Verskeie moontlike patogene was uiteindelik as opportunistiese patogene geklassifiseer, soos byvoorbeeld *Enterobacter sakazakii* and *Ent. agglomerans*.

Polymerasekettingreaksie (PKR) is 'n nuwe, alternatiewe metode van organisme-identifikasie naas konvensionele mikrobiologiese ontledings en is baie gerieflik om te gebruik. Melk was egter nie 'n maklike medium vir die PKR-tegniek nie. Daar is tydens die studie probleme ondervind met herhaalbare teiken-DNA. Die PKR-produkgroottes het nie altyd met verwagte groottes gekorrelleer nie.

Plaaslike stamme sal getoets moet word en die PKR-voorstelling van plaaslike stamme sal gereeld gekorreleer en op datum gebring moet word.

Daar is bevind dat sekere faktore die PKR-resultate beïnvloed het. Bevriesing en ontdooiing van monsters het 'n negatiewe invloed op PKR-resultate gehad. Die Dynabead® DNA-konsentrasiesisteem was effektief om lae selkonsentrasies uit te wys, maar is ook deur die vries/ontdooi-proses beïnvloed. Isolering van *L. monocytogenes* met konvensionele en PKR-metode het ooreengestem. Opsporing van *Campylobacter* en *Brucella* in voedsel met die PKR-metode het belowend gelyk. Alhoewel sekere probleme nog uitgeklaar moet word, bly dit 'n gerieflike tegniek om in die toekoms te gebruik om patogene vinnig op te spoor en te identifiseer.

As gevolg van verskeie redes word antibiotika soms foutiewelik deur boere aan siek diere toegedien. Onvoldoende kennis en die gebruik van antibiotiese middels, wat groei bevorder, het tot gevolg dat antibiotikabestande mikro-organismes in die omgewing beland. In die studie is die teenwoordigheid van antibiotikabestande gene in organismes, wat in melk, groentes en uit die omgewing geïsoleer was, ondersoek. Die bestandheidsprofiele van staphylococci, in 1997 en 1998 uit rou en gepasteuriseerde melk geïsoleer, toon dat die stamme wat weerstandig teenoor drie en meer antibiotikas was, bestand teen streptomycin, tetracycline en cephalosporin was. Die meerderheid van koagulase-negatiewe staphylococci (KNS) was bestand teen ciprofloxacin, streptomycin en oxacillin.

Melk is 'n voedingryke produk, wat teen 'n billike prys, algemeen beskikbaar is. In 'n land waar die meerderheid van die gemeenskap in armoede leef, is dit 'n baie belangrike voedingsbron. Melk wat direk by die boer of by depots gekoop word, is meestal goedkoper as klaar verpakte melk wat deur supermarkte verkoop word. Uit die studie was dit duidelik dat díe melk nie aan die wetlike standaarde voldoen het nie. Patogene en antibiotikabestande stamme was wel teenwoordig. Die verbruiker het die reg om 'n hoë-kwaliteit melk, wat vry van patogene en antibiotikaresidue is, te geniet.

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To my husband, Deon and my children, Deon and Elani

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LIST OF ABBREVIATIONS

Antibiotics

AMP	Ampicillin
AMOX/CA	Augmentin (Amoxycillin/clavulanic acid)
CTAX	Cefotaxime
CTIN	Cefoxitin
CTRX	Ceftriaxone
CHL	Chloramphenicol
CIP	Ciprofloxacin
SMX/TMP	Cotrimoxazole
ERY	Erythromycin
GEN	Gentamicin
IMIP	Imipenem
KAN	Kanamycin
OXAC	Oxacillin
PEN	Penicillin
RIF	Rifampicin
STR	Streptomycin
TET	Tetracycline
VAN	Vancomycin

General

C.	*Citrobacter*
CNS	Coagulase negative staphylococci
cfu	*Colony forming units*
Ent.	*Enterobacter*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
Erw.	*Erwinia*
E.	*Escherichia*
HUS	Haemolytic uraemic syndrome
HTST	High temperature, short time process
K.	*Klebsiella*
LP	Lactoperoxidase
LF	Lactose fermenting
Lecl.	*Leclercia*
MPC	Magnetic Particle Concentrator
MRSA	Methicillin-resistant *S. aureus*
MWM	Molecular weight marker

(List of abbreviations continued)

NCCLS	National Committee for Clinical Laboratory Standards
NLF	Non lactose fermenting
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
Prov.	*Providencia*
Ps.	*Pseudomonas*
S.	*Salmonella*
SS agar	*Salmonella Shigella* agar
Ser.	*Serratia*
Sh.	*Shigella*
Str.	*Streptococcus*
TCN	Theoretical cell numbers
tntc	Too numerous to count
VTEC	Verotoxigenic *E. coli*

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The incidence of foodborne diseases is generally underestimated as a) the time from onset and duration of symptoms may complicate diagnosis and b) the time taken to demonstrate a causative agent in suspected food, if still available for analysis, may be lengthy (Hill, 1996).

1.1 BACTERIA ASSOCIATED WITH MILK SPOILAGE AND INFECTIONS

Bacteria present in milk products can act as invasive or opportunistic pathogens of man and animals. Milk can be contaminated by numerous routes, some being readily identifiable such as faecal contamination and udder infections. A major area of concern is obviously mastitis control. Mastitis is the infection of mammary glands of cows giving rise to the presence of specific bacteria in milk. Infected glands cause bacteria to be excreted directly into the milk. Results of mastitis include a) decreased milk production and labour, b) costs of veterinary treatment and antibiotics and c) further losses after treatment until the milk is cleared of any antibiotic residue (Saran, 1995). Drugs can still be secreted eight to nine days after dosing (Ziv *et al.*, 1995). Pathogenic organisms causing mastitis include *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysagalactiae.* Environmental organisms causing sub-clinical mastitis are *Escherichia coli* and coliform enteric bacteria (Saran, 1995). Treatment of mastitis is also problematic concerning tissue penetration and the effect of the milk on the bacteria. In a study by Louhi-Lehtiö *et al.* (1994), where it was found that when nutrient broth was replaced with milk as a test medium, the antibacterial activities of trimethoprim-sulphadoxine, spiramycin and ampicillin were significantly decreased.

Other routes of milk contamination are transient causes which include contamination of milk by the handler, and environmental sources such as dust, water and residues in improperly cleaned equipment. The drinking water given to herds should be free of organic material and silt (Horner, 1986).

1.1.1 Staphylococci:

Staphylococcus aureus is secreted into milk from cows in which it is the major causative organism of mastitis. Most staphylococcal udder infections originate in the teat canal at which stage the somatic cell count is still normal (Du Preez, 1988). Certain strains of *S. aureus* produce heat stable enterotoxins that can give rise to vomiting and less frequently, diarrhoea. Human carriers of *S. aureus* play an important role in the spreading of the organism as a foodborne pathogen (Collins, Lyne & Grange, 1995; Varnam & Evans, 1991).

S. aureus is difficult to treat with antibiotics, especially during lactation. Contributing factors are poor penetration of the drugs to enter infected tissue, bacterial resistance and reduced antimicrobial activity in a milk medium. In a study done in Trinidad in 1993, 34percent of *S. aureus* strains were resistant to penicillin and ampicillin and, to a lesser extent, methicillin with 53.6 percent of strains being enterotoxigenic (Adesiyun, 1994).

S. caprae found in goat's milk, tend to be more resistant to antibiotics than other staphylococcal species and may give rise to endocarditis and urinary tract infections in humans (Vandenesch *et al.,* 1995).

1.1.2 Streptococci and enterococci:

Group B streptococci are causative agents of bovine mastitis. In humans, group B streptococci can cause septicaemia and multiple infections in neonates (Bopp & Lämmler, 1995; Rivas *et al.*, 1997). *Streptococcus agalactiae* is an obligate parasite of the bovine mammary gland causing low grade, persistent infections in these glands (Keefe, Dohoo & Spangler, 1997).

d) Verotoxigenic *E. coli* (VTEC) producing strains can cause haemorrhagic colitis and HUS (Sharp *et al.*, 1994). More than 50 percent of verocytotoxin-producing *E. coli* are of serotype 0157:H7 being transmitted from person-to-person, animal-to-person, and may be present in raw milk, contaminated pasteurised milk and untreated water (Calman, 1995; Sharp *et al.*, 1994; Travena *et al.*, 1996). Cattle appear to be a primary reservoir of *E. coli* 0157:H7 with beef and milk produce as the most important causative agents in outbreaks due to poor hygienic practises during slaughtering and milking (Humphrey, 1997).

Salmonella species are natural inhabitants of the intestinal tract of animals/birds and may be isolated from water that has been contaminated with faecal material. For years salmonella species have been recognised as major cause of enteric diseases as a result of food poisoning (Varnam & Evans, 1991).

Enteric fever (typhoid fever) strains of *Salmonella* on ingestion reach the small intestine from where they may enter the lymphatic system and the blood stream. After ten to fourteen days, fever, malaise, bradycardia and myalgia occur, the mortality rate being ten to fifteen percent of treated cases (Carlin, Nguyen-the & da Silva, 1995; Eley, 1992). In California, *S. dublin* is more likely to be isolated from raw milk than any other serovar with ten to twenty percent of hospitalised cases being reported as fatal (Fang, Araujo & Geurrant, 1991). Strains of *Salmonella* may cause enterocolitis, giving rise to diarrhoea, nausea, vomiting, intestinal mucosa damage and, in two to four percent cases, bacteraemia may result after ingestion of contaminated food (Varnam & Evans, 1991).

It is usually considered that very high numbers of *Salmonella* ($>10^5$ cfu) are required to initiate an infection, although food with low doses (10^2 cfu) has been reported to give rise to outbreaks of food poisoning (Collins, Lynne & Grange, 1995). *Salmonella* originally excreted in milk from infected cattle may survive for two weeks in cultured milk products (Fang, Araujo & Guerrant, 1991).

Antibiotic treatment is not desirable in treating *Salmonella* enteritis, but it is advocated when it is associated with septicaemia. *Salmonella* strains that show

multiple resistance to antibiotics are increasing and a cause for concern (Varnam & Evans, 1991).

1.1.4 *Yersinia*:

Yersinia haemolitica and *Yersinia multocida* produce diseases in cattle, and transmission to humans can be prevented by drinking pasteurised instead of raw milk (Fang, Araujo & Guerrant, 1991). Infections in humans after ingestion are diarrhoea, but in some patients adverse immunological reactions, pneumonia, meningitis or sepsis may result as complications one or two weeks after onset (Varnam & Evans, 1991). *Y. haemolitica* produces a heat stable toxin similar to the enterotoxin produced by ETEC (Harnett, Lin & Krishnan, 1996).

1.1.5 *Campylobacter*:

Recently, *Campylobacter jejuni* has surpassed *Salmonella* as the most common agent of diarrhoeal disease and gastro-enteritis (Illingworth, 1992; Lior, 1996). Gastro-enteritis with prolonged symptoms, the consequent malabsorption of nutrients and even complications such as HUS, biliary tract infections, cholecystitis and hepatitis, do occur (Varnam & Evans, 1991).

Consumption of raw milk or post-pasteurised contaminated milk constitutes a major risk for contracting *C. jejuni* that has the capacity to invade systemically. A "*Campylobacter*-positive cow" would most probably contract *Campylobacter* by drinking contaminated water. Milk could be contaminated with *Campylobacter* through mastitis or faecal contamination (Humphrey, 1997). Only very low levels of contamination in milk are required to cause infection in humans. The organism is, however, heat-sensitive and the pasteurisation process normally controls transmission by milk (Humphrey, 1997).

1.1.6 *Listeria*:

Listeria monocytogenes is increasingly being recognised world-wide as a foodborne pathogen (Harvey & Gilmour, 1994). Maura, Destro and Franco (1993), found that *Listeria* is more frequently present in raw milk than in pasteurised milk.

Before the 1980s it was believed to be an animal pathogen (Varnam & Evans, 1991). *L. monocytogenes* causes listeriosis that manifests as an influenza-like condition (Post, 1996). Although awareness has increased, problems are still evident. Infections caused by *Listeria* are often present with non-specific symptoms preventing a definite clinical diagnosis. Meningitic, septicaemic and oculoglandular listeriosis are complications that can occur in people of all ages (Varnam & Evans, 1991). In healthy people, listeriosis does not usually develop beyond the enteric phase. In neonates, respiratory distress and heart failure are some of the complications that may occur. Abortion or still birth in pregnant women has also been reported (Post, 1996).

1.1.7 Bacillus:

Bacillus cereus is widespread in the environment and contamination during processing can cause spoilage of milk, and toxin production. Although the vegetative bacterial stage is the most prevalent in raw milk, spores of *Bacillus* species are resistant to heat and can survive pasteurisation. Spores can resist temperatures up to 100°C. *B. licheniformis and B. cereus* are commonly found in milk at all stages of processing. *B. cereus* in milk powder has been associated with outbreaks of foodborne disease and can contaminate other milk-based products such as baby foods (Crielly, Logan & Anderton, 1994). *B. cereus* can produce two types of toxins and gastro-enteritis occurs as an emetic or a diarrhoeal syndrome depending on the toxin formed (Scharft, Laberge & Griffiths, 1997).

1.1.8 *Aeromonas*:

Aeromonas species are found in twenty percent of raw milk and cheese samples and cause acute diarrhoea (Pin *et al.*, 1992). *A. hydrophila* grows at 37°C and it is recorded that the hydrophila group can cause foodborne gastro-enteritis (Collins, Lynne & Grange, 1995). In susceptible persons *Aeromonas* can give rise to complications such as septicaemia and meningitis (Varnam & Evans, 1991).

1.1.9 *Brucella*:

Cattle are the most important hosts of *Brucella abortus* and goats of *B. melitensis.* Animals infected with *Brucella* may secrete the pathogen in milk, although no clinical symptoms are present (Leal-Klevesas, 1995). Brucellosis is an occupational disease affecting farmers, abattoir workers and veterinarians. Other individuals become infected when contaminated raw milk is consumed. The incubation period in humans is one to six weeks with an onset of insidious fever, weakness and aches. The fever is undulating and there may be gastro-enteritis and symptoms of the nervous system. Hepatitis and osteomyelitis may result. Following the initial infection, a chronic stage may develop that is difficult to diagnose clinically (Varnam & Evans, 1991). Prevention of brucellosis in milk can be achieved by adequate pasteurisation (Varnam & Evans, 1991).

1.1.10 Mycobacteria:

Mycobacterium paratuberculosis is excreted in milk from infected cows or asymptomatic carriers. Johne's disease or chronic ileocolitis results in diarrhoea and, finally, death in cattle. A positive association has been shown between *M. paratuberculosis* and Crohn's disease in twenty to 38 percent of human patients (Chiodini & Hermon-Taylor, 1993). *M. bovis* contracted from contaminated raw milk can cause tuberculosis in humans, which is clinically indistinct from *M. tuberculosis* (Hardie & Watson, 1992).

1.2 PREVENTION OF THE DISSEMINATION OF HAZARDOUS BACTERIA FROM MILK

Good management and healthy hygienic practices on farms are extremely important to maintain healthy dairy herds and to ensure milk of a good quality. This can be achieved by a) disinfecting the parlour and equipment to prevent bacterial build-up; b) udders should be stringently cleaned prior to milking,; and c) feed should be free of contamination (Horner, 1986; O'Toole, 1995). Once collected, milk should be stored and transported to the processing plants with appropriate precautions taken to ensure minimal bacterial proliferation (Saran, 1995).

Milk has a natural inhibitory system that can influence milk quality in that inhibitors may mask the true bacteriological condition of the milk (O'Toole, 1995). According to Muir (1996), the lactoperoxidase system in milk has the ability to inhibit the growth of lactic acid bacteria, coliforms, salmonellas, shigellas and pseudomonads. Growth of species of *Yersinia* can also be controlled by the lactoperoxidase system (O'Toole, 1995). However, it has been found that the effectiveness of the lactoperoxidase system is subject to pH inactivation; at pH 7.5 the isolation of the pathogen *C. jejuni* was improved (O'Toole, 1995). Other natural inhibitors in milk are lysozyme and lactoferrin (Banwart, 1989; O'Toole, 1995). These natural inhibitory substances can cause problems in the isolation of bacteria as they may partially affect the bacteria cell, which remains in a semi-dormant state. These cells may not be traceable by means of conventional microbiological methods, but are still viable to cause disease.

The quality of milk depends on the microbiological status of the milk as distributed to the processor and consumer. Legislatory standards for dairy products in South Africa are incorporated in Act 54 of 1972, the Foodstuffs, Cosmetics and Disinfectants Act, Regulation 1555 of 21 November 1997. The maximum microbiological content of raw milk for processing, and pasteurised

milk as stipulated, pertains to general numbers of bacteria, coliform bacteria and *Escherichia coli*.

The ideal would be that all milk and milk produce should be batch-tested and passed totally free of any pathogens before being distributed and consumed. Pasteurisation is performed to ensure this. Regulations regarding pasteurisation are as follows: a) the batch method; 63°C for thirty minutes; or b) the high temperature short time process; 72°C for fifteen seconds with cooling to below 5°C within thirty minutes. Pasteurised milk should be kept at a temperature of less than 5°C during distribution and storage. Drinking untreated raw milk can be a dangerous health risk, although some communities knowingly prefer to continue doing so.

Even under beneficial conditions, it is impossible to eliminate milk contamination entirely. It should be noted that outbreaks of food poisoning have been attributed to poor plant design that can cause cross-contamination of treated milk by raw milk. It can also be ascribed to untrained operators and complacency (Eley, 1992; Gilmour & Harvey, 1990). The emergence and recent recognition of *Listeria* as a pathogen has resulted in the increase of the severity of pasteurisation conditions in some processors (Moura, Destro & Franco, 1993).

1.3 VEGETABLES AS A SOURCE OF HAZARDOUS BACTERIA

Due to a world-wide change in diet, fresh and minimally processed foods, especially vegetables, have become more likely to be vehicles for the dissemination of bacterial pathogens (Hedberg, MacDonald & Osterholm, 1994). Bacteria are usually found on the surface of vegetables. Most of the bacteria present after harvesting originate from the soil. Rain and insects can act as vehicles for bacterial transfer among field plants. Humidity influences post-harvesting bacterial survival and spreading, as conditions of drought have the effect of lowering bacterial numbers (Lund, 1992). Nguyen-the and Carlin (1994) have concluded that the incidence of foodborne pathogens is lower in vegetables than in dairy and meat produce.

Sources of contamination with the pathogens *Salmonella, E. coli,* and *L. monocytogenes* are: manure, polluted irrigation water, animals, birds and unhygienic post-harvesting practices (Lund, 1992). *B. cereus* spores from soil can contaminate vegetables. In Chile a high incidence of *S. typhi, Vibrio cholerae* and *Helicobacter pylori* were responsible for enteric infections due to the consumption of contaminated vegetables. Untreated sewage water was used for irrigation (Hopkins *et al.*, 1993).

1.3.1 *Escherichia. coli:*

E. coli 0157:H7 infections have resulted from garden manure (Cieslak *et al.*, 1993; Travena *et al.,* 1996). *E. coli* was isolated from 25 percent of pre-packed, shredded, mixed vegetables sold in supermarkets in England (Brocklehurst, Zaman-Wong & Lund, 1987).

1.3.2 *Salmonella:*

Salmonella species have been implicated in numerous outbreaks of food poisoning from many different vegetable sources as they have a) ubiquitous distribution in the environment; b) are extremely adaptable in their growth requirements and thus are capable of survival in adverse conditions; and c) may possess virulence factors (D'Aoust, 1994). Levels of salmonella in fresh vegetables can range from nearly two to eight percent. A single report from Italy revealed that 69.9 percent of lettuce and fennel samples had been contaminated with salmonella, and that consumption of these products in their raw state had led to outbreaks of salmonellosis (D'Aoust, 1994). Tomatoes have been identified as a vehicle of *S. javiana* and *S. montevideo* foodborne disease (Hedberg, MacDonald & Osterholm, 1994). Spices such as pepper, parsley, coriander and peppermint have been involved in the outbreak of salmonellosis (Gustavsen & Breen, 1984).

1.3.3 *Shigella*:

Shigellae are pathogenic organisms found in human faeces and in the environment (Eley, 1992). *Shigella* is rarely isolated from animals. The mechanism of pathogenesis is invasion of the mucosa of the illium or colon causing blood in the stools. Another mechanism is the production of an enterotoxin called Shiga toxin. Presence of the toxin in the bowl, induces intestinal tissue secretions with diarrhoea and, if not treated, dehydration and eventually death, can result (Eley, 1992). *Sh. sonnei* isolated from commercially distributed, shredded lettuce, had been reported to be responsible for an outbreak of shigellosis (Lund, 1992).

1.3.4 *Yersinia*

Non-pathogenic strains of *Yersinia enterocolitica* have been isolated from minimally processed fresh vegetables in up to 76 percent of samples tested (Nguyen-the & Carlin, 1994).

1.3.5 *Campylobacter*

Poor sanitary conditions during cultivation or harvesting have been incriminated as sources for the contamination of vegetables by *Campylobacter* species (Nguyen-the & Carlin, 1994).

1.3.6 *Listeria*

Listeria monocytogenes, an organism widely distributed in the environment, can contaminate foods during production and processing. In a study by De Simón, Terrago and Ferrer (1992), *L. monocytogenes* was isolated from 7.8 percent of vegetable samples investigated. Prepacked mixed salads and raw vegetables, such as lettuce, cabbage, broccoli and cauliflower, were responsible for outbreaks of listeriosis (Carlin, Nguyen-the & da Silva, 1995; Lund, 1992). The

L. monocytogenes content on endive leaves has been seen to increase a hundred-fold at 10°C in four days, although the leaves did not show any signs of spoilage (Carlin, Nguyen-the & da Silva, 1995). Only after another seven days at 10°C, extensive spoilage on leaves was visible due to a further increase in *L. monocytogenes* numbers. Raw vegetables in the form of coleslaw have been associated with outbreaks of listeriosis in the USA (Sizmur & Walker, 1988). The survival of *Listeria* at 4°C has been studied and it was found that viable counts could increase two-fold in a period of four days (Sizmur & Walker, 1988).

1.3.7 *Bacillus*

Bacillus cereus has been isolated from vegetable seed and seed sprouts (Nguyen-the & Carlin, 1994). Sometimes spices and cereals with high carbohydrate content may be responsible for gastro-enteritis due to *B. cereus* (Jay, 1992).

1.3.8 *Aeromonas*

Cytotoxic and haemolytic *Aeromonas* species have been associated with vegetables in the USA (Nguyen-the & Carlin, 1994).

1.3.9 *Vibrio cholerae*

Vibrio cholerae has been found to be responsible for outbreaks of food infections due to the consumption of raw vegetables and salads pre-contaminated by irrigation with polluted water or fertilised with manure (Nguyen-the & Carlin, 1994).

1.3.10 *Clostridium*

Clostridium perfringens has been isolated from partially processed vegetables, runner beans, potatoes, carrots and beetroot. *Clostridium botulinum* has been reported to be responsible for three outbreaks of type A botulism in potato salad and thirty cases of type B botulism after consumption of commercially prepared chopped garlic in soybean oil (Lund, 1992).

1.3.10 *Citrobacter freundii*

A verotoxinogenic *Citrobacter freundii* strain that originated from organic garden manure, caused the contamination of parsley which was consumed in the form of green butter (Tschäpe *et al.,* 1995).

1.4 PREVENTION OF THE DISSEMINATION OF HAZARDOUS BACTERIA FROM VEGETABLES

The risk of acquired a foodborne disease from vegetables depends on the type of food, its source, its preparation or handling and the host's resistance to the infectious agent. Numerous different vegetables and bacterial species have been associated with food poisoning. There is very little legislation concerning the microbiological content of vegetables and no standard processing recommendations have been suggested. Storage in refrigerators can actually enhance the growth of psychrotrophic, foodborne pathogens such as *L. monocytogenes* (Lund, 1992). Vegetables or salad ingredients are not often investigated as primary sources of infection and microbiological results are often delayed. In fact, vegetables may not even be considered or implicated as source at all, a situation which requires rectification, especially in South Africa where faecal contaminated water may be used for crop irrigation.

1.5 USE OF ANTIBIOTICS IN VETERINARY SCIENCE

1.5.1 Non-therapeutic Usage

In the late 1960s a committee on the use of antibiotics in animal husbandry and veterinary medicine recommended the use of growth-promoting antimicrobial agents, stating that the efficiecy of therapeutic drugs would not be impaired by the development of resistance (Wray, 1997). It is believed, however, that sub-therapeutic amounts of antimicrobial agents in animal feed used for growth promotion and disease prevention are responsible for the accumulation of resistant bacteria affecting humans (Holmberg *et al.,* 1984). According to Bordas *et al.* (1997), the pesticides, carbaryl, captan and malathion, and combinations of antibiotic residue, have been detected in food of animal origin and in milk samples, although most were at levels below established tolerances (Bordas *et al.,* 1997). In the early 1990s vancomycin-resistant enterococci had been detected in the UK, Denmark and Germany with strong indications that the use of avoparcin, a glycopeptide used as a growth promoter, had been responsible for the increased resistance observed (Bates, Jordens & Griffiths, 1994.). Apramycin is an aminoglycoside antibiotic that was licensed for veterinary use only. Certain bacteria, however, have the ability to produce an aminoglycoside-modifying enzyme which not only inactivates apramycin, but also other aminoglycosides like gentamicin which is used in human therapy (Johnson, 1997). Apramycin-gentamicin-resistant strains of enterococci in humans have been isolated in European countries (Johnson, 1997).

1.5.2 Use of Antibiotics in Controlling Mastitis in Dairy Herds

Mastitis causes higher numbers of specific pathogenic bacteria in milk. Milk contains substances that are inhibitory to some bacteria, but their activities may not have restricted infections or outbreaks of foodborne infections effectively (O'Toole, 1995). *Staphylococcus aureus* and *Streptococcus agalactiae* are the two major agents of bovine intra-mammary infections (Rivas *et al.*, 1997). Other

bacteria causing mastitis are *E. coli*, *Klebsiella*, and species of *Pseudomonas* and *Yersinia* (Kaartinen *et al.*, 1995).

There are therapeutic problems involved in the eradication of bacterial mastitis, such as the poor penetration of antibiotics in mammary tissues and the fact that the distribution of agents in swollen udders is limited (Pyörälä, Kaartinen & Käck, 1994). In addition, antibiotics may also exhibit reduced activity due to interfering factors in the milk. Studies have shown that antibacterial susceptibility methods used *in vitro* do not necessarily simulate the *in vivo* conditions, explaining why therapy for mastitis often fails (Louhi-Lehtiö *et al.*, 1994).

A further administrative aspect that must be considered, is the requirement for antibiotic exclusion before milk can once again be collected for distribution. Tilmicosin, for example, rapidly penetrates from blood to milk, but has a slow elimination time from the milk. After eight to nine days, tilmicosin is still secreted in the milk (Ziv *et al.*, 1995).

1.5.2.1 Staphylococcal mastitis

A number of studies have been performed to evaluate the efficiency of antibiotics for mastitis control. In a study conducted on *S. aureus* strains isolated from mastitic milk, 34 percent were resistant to one or more of the following antimicrobial agents: penicillin, ampicillin and methicillin (Adesiyun, 1994). In another study the effectiveness of some antibiotic groups used in human medicine were investigated. They were penicillin, cephalosporin, erythromycin, ciprofloxacin, norfloxacin, rifampicin, pirlimycin (a lincosamide) and tilmicosin (a macrolide). Rifampicin was the only antibiotic tested that eliminated *S. aureus* completely. Recommendations made for the emergence of rifampicin resistance were the combined use of rifampicin and norfloxacin or cloxacillin (Owens, Ray & Washburn, 1993).

1.5.2.2 Coliform mastitis

Mastitis caused by the coliform bacteria for example *E. coli*, *Klebsiella* and *Pseudomonas,* are exceptionally difficult to treat due to drug resistance and lack of effective penetration of appropriate antibiotics into udders or milk (Kaartinen *et al.*, 1995). In an experimentally induced *E. coli* mastitis, two drug therapy methods were used, a trimethoprim-sulfonamide combination and colistin. No significant differences occurred in bacterial counts for the different therapy groups and the control group (Pyörälä, Kaartinen & Käck, 1994). Fluoroquinolones have been advocated for *E. coli*, *Klebsiella* and *Pseudomonas* mastitis by Kaartinen *et al.* (1995), and different administration techniques were assessed. When antibiotics were given intramuscularly and subcutaneously, delayed absorption was experienced which also limited elimination. This gave opportunity for the sustained release of the antibiotic at the injection site. One can assume that this can only lead to small amounts of antibiotic release over a long period, resulting in the continued presence of antibiotics in the milk. Such therapeutic practices induce the development of antibiotic resistance in bacteria and emphasise the necessity to ensure that the animal is proclaimed clean before further milking for collection can proceed. Cefquinome, a broad-spectrum cephalosporin that resists ß-lactamases, has been administered to *E. coli* mastitic cows, resulting in a significantly improved recovery compared to ampicillin and cloxacillin treatment. Return to normal milk production can thus be much sooner (Shpigel *et al.,* 1997).

It is evident that a wide range of anti-microbial groups are constantly being assessed for the treatment of bovine mastitis and many of these antibiotics are considered as vital last-line treatments for human infections.

The role of once human reserved antibiotic groups as veterinary drugs and the spreading of bacteria, carrying antibiotic-resistant genes from animals to man is still debated, but is becoming an irrefutability. Transmission of antibiotic-resistant strains due to the injudicious use of antimicrobial agents in animals from farms into the community, and subsequently into hospitals, has been

reported to occur, and has been shown to have a detrimental effect on human health (Ombui, Macharia & Nduhiu, 1995).

The use of antibiotics for veterinary medicine, prophylactics or growth promotion and feed preservation will certainly influence the prevalence of antibiotic-resistant bacteria that can enter the food chain. It is of major importance to a) assess the prevalence of resistant bacteria that can be transmitted from farms; and b) control antibiotic usage, or the efficiency of antimicrobial agents will be seriously compromised.

1.6 DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN BACTERIA

Antibiotic resistance develops in bacteria as they have a high mutation rate and rapid life cycle. Many genera possess the ability to acquire genetic material from external sources (Berkowitz, 1995). The spreading of resistant bacteria from different sources and the entering of the environment are facts currently causing concern.

1.6.1 Mechanisms of Resistance

There are four main mechanisms by which resistance in bacteria can be obtained, namely, a) prevention of the drug reaching the target; b) rapid extrusion of the drug; c) enzymatic modification of the drug; and d) alteration of the molecular target of the drug, as described by Berkowitz (1995). If more than one mechanism is at work, an increased degree of resistance can result.

1.6.1.1 Barrier to entry

An important mechanism of resistance in gram-negative bacteria is the lipopolysaccharide outer membrane, that is capable of altering the degree of entry of the antibiotic, leaving the bacteria unaffected (Berkowitz, 1995).

1.6.1.2 Rapid extrusion

The ability of bacteria to pump antibiotics out of the cell results in the non-accumulation of the antibiotic or the accumulation of only subinhibitory concentrations in the cell. Aminoglycosides enter the cell at a very slow rate and are actively extruded by the plasmid-encoded proteins located in the cell membrane (Davis *et al.,* 1990). Active efflux is best recognised as a mechanism of tetracycline and aminoglycoside resistance, but is also applicable to other antibiotics, such as the macrolides, fluoroquinolones and chloramphenicol (Berkowitz, 1995).

1.6.1.3 Enzymatic modification

Modification enzymes are plasmid- or chromosomal-encoded. This resistance mechanism is important against the ß-lactam antibiotics, aminoglycosides and chloramphenicol.

The enzyme ß-lactamase hydrolyses the ß-lactam bond of penicillins and cephalosporins, inactivating the drugs. Examples of gram-negative bacteria capable of producing ß-lactamases are the members of *Enterobacteriacea, Pseudomonas spp., Xanthomonas maltophilia* and *Acinetobacter spp.* (Berkowitz, 1995). Some gram-negative bacteria, *Enterobacter*, *Serratia*, *Morganella morganii* and *Pseudomonas aeroginosa,* have the ability to produce two groups of ß-lactamases, one being chromosomal-encoded and the other group plasmid-encoded. The ß-lactamase genes in unchallenged cells can be repressed, but when the bacterium is exposed to certain ß-lactams, even at subinhibitory concentrations, genes may be derepressed and the enzyme is produced in large quantities (Berkowitz, 1995).

Aminoglycoside-modifying enzymes are very specific and are attached to the cytoplasmic membrane. The enzymes are encoded on transposons or plasmids, modifying and inactivating the aminoglycosides by phosphorylation, acetylation or adenylation (Hentges, 1995). Aminoglycosides in clinical use in South Africa

are: gentamicin, tobramycin, kanamycin, streptomycin and amikacin, which is a semisynthetic derivate of kanamycin (Davis *et al.*, 1990).

1.6.1.4 Target alteration

Penicillin-binding proteins (PBP's) are enzymes involved in cell wall synthesis, and are responsible for forming cross-linkages within the peptidoglycan layers of the cell wall. If the PBP's are not available for maintaining cell wall integrity due to the binding of ß-lactams to the active site, cell lysis and death can result (Berkowitz, 1995).

1.6.2 Antibiotic Resistance Development in Specific Bacteria

1.6.2.1 *Staphylococci*

In Finland (1994), seven percent of 168 strains of coagulase-negative staphylococci (CNS) isolated from milk, were ß-lactamase positive (Honkanen-Buzalski, Myllys & Pyörälä, 1994). The inflammation caused by the coagulase-negative staphylococci (CNS) in the udder was not very serious, but many of the strains were resistant to several antibiotics, of which resistance to penicillin, ampicillin, erythromycin, tetracycline, and chloramphenicol were evident. Although CNS bacteria are not a direct threat to human health, their ability to disseminate antibiotic-resistance determinants does pose problems for future therapeutic options (Honkanen-Buzalski, Myllys & Pyörälä, 1994). *S. aureus* strains from bovine mastitic milk isolated in the West Indies have been found to be resistant to penicillin (23.6%), ampicillin (17%) and methicillin (1.2%) (Adesiyun, 1994). Methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant CNS have been isolated from clinical samples (Berkowitz, 1995 & Steinberg *et al.*, 1997). Vancomycin is at this stage the last available drug for many staphylococcal infections in the USA, however, vancomycin-resistant strains of *S. epidermidis* have been reported (Berkowitz, 1995).

Rifampicin has been used in a study against *S. aureus* infected mammary glands. Rifampicin penetrates the leukocytes and has been effective against slow metabolising bacteria. Initially it was most effective in killing *S. aureus,* but during the course of treatment resistance to the drug was reported (Owens, Ray & Washburn, 1993).

In addition to described mechanisms of resistance (Section 1.6.1.), the ability of *S. aureus* to bind with milk fat globules and its capacity to form capsules, has been shown to increase resistance to penicillin-G (Ali-Vehmas *et al.,* 1997).

1.6.2.2 *Streptococci* and enterococci

In a survey in Italy conducted from 1993 to 1995, resistance of *Streptococcus pyogenes* to erythromycin was seen to increase sharply (Cornaglia *et al.*, 1996). Enterococci have a natural tolerance to ß-lactams, cephalosporins, lincosamides and polymyxins. Of great importance is their acquired resistance to the aminoglycosides, tetracyclines, macrolides, chloramphenicol and penicillins. At this stage enterococci isolated from milk and milk products in Italy show higher sensitivity to most antibiotics compared with clinical strains (Giraffa, Carminati & Neviani, 1997).

1.6.2.3 *Enterobacteriacea*

E. coli and salmonella from natural environments and a variety of food products are becoming progressively more resistant to antibiotics (D'Aoust, 1994). *Klebsiella pneumoniae* and *K. oxytoca* are responsible for nosocomial infections and show multiple resistance to a variety of antibiotics. Resistance genes aimed at inactivating aminoglycosides that were originally found in *Klebsiella* have subsequently appeared in isolates of *E. coli* and *Citrobacter freundii* (Jones *et al.,* 1997).

Ombui, Macharia & Nduhiu (1995) studied antimicrobial resistance and plasmid profiles of *Escherichia coli.* They found that 95 percent of the strains were resistant to sulphamethoxazole and, to varying degrees, to other antibiotics,

including ampicillin, tetracycline, cotrimoxazole, streptomycin, kanamycin, gentamicin and chloramphenicol, with 29 percent strains showing multiple resistance (Ombui, Macharia & Nduhiu, 1995). No one particular molecular weight plasmid could be implicated as being responsible for a specific resistance type with resistance genes being recorded across various plasmids and present in strains that did not contain plasmids (Ombui, Macharia & Nduhiu, 1995).

In the treatment of salmonellosis, multiple drug-resistant strains have accounted for an increasing percentage of salmonella infections. Two years after apramycin was licensed for veterinary use in the UK, resistance was detected in *Salmonella* species (Wray, 1997).

Strains of *Salmonella* and *Shigella* species showing resistance to two or more antibiotics have been isolated, especially in countries with poor sanitation. *Salmonella* spp. with resistance to ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole and *Shigella* spp. with ampicillin and trimethoprim-sulphamethoxazole resistance have been detected (Berkowitz, 1995). It is known that *Shigella* rapidly acquires antibiotic resistance, that is often plasmid-mediated (Eley, 1992).

1.6.2.4 *Yersinia* and *Campylobacter*

Yersinia which is resistant to ß-lactams, streptomycin and tetracycline and *C. jejuni* exhibiting increased resistance to quinolones have been described (Chaslus-Dancla *et al.*, 1995; Lior, 1996).

1.6.3 IMPLICATIONS OF BACTERIAL RESISTANCE DEVELOPMENT

Antibiotic resistance mediated by target alterations is usually determined by the bacterial genome due to mutations or the acquisition of new genetic material, which can occur by means of three mechanisms, namely conjugation, transformation and transduction. Resistance generated by mutation is often attributed to continuous exposure to low levels of antibiotic, as would be fulfilled in the environment if antibiotic usage is not contained (Berkowitz, 1995). Resistance attributed to enzyme modifications and extrusion are generally determined by genes carried on transposons or plasmids. Such resistance determinants therefore exhibit a potentially high rate of dissemination by virtue of the genetic elements having broad bacterial host ranges (Berkowitz, 1995).
Once antibiotic-resistant gene pools have evolved, they are often long-lived and the situation is extremely difficult to reverse. There are many health risk factors involved both directly and indirectly in the handling and consumption of food produce containing pathogenic and non-pathogenic bacteria.

1.7 AIM OF THE STUDY

The present study was designed to investigate a) the bacterial content of milk and vegetables distributed in the Bloemfontein area; b) assess conventional and polymerase chain reaction methods for bacterial detection/identification; and c) to determine the degree of antibiotic resistance that is currently present in the environment pertaining to bacteria isolated from milk and vegetables.

CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

2.1.1 Milk

Pasteurised milk was collected from different types of processing units, large industrial processing plants, small-scale industries and farm/home industries. The small-scale industries have town depots as distribution outlets. Milk was also obtained from individual cows on the farms. Samples were taken in 1997 on 11/02: 4 industries and 4 depots; 03/03: 6 cows, 3 raw batches and 1 depot; 21/04: 6 cows, 2 raw batches and 3 depots; 12/05: 3 raw batches and 1 depot; 20/10: 6 raw batches and 5 depots and in 1998 on 09/03: 6 raw batches and 5 depots. Samples were taken aseptically and held on ice until arrival at the laboratory. For conventional microbiological analysis tests were initiated the same day, PCR analysis milk was centrifuged [section 2.5] and held at –70°C and for antibiotic resistance studies [section 2.6], glycerol was added to 10% final volume mixed, and samples were held at -70°C.

2.1.2 Vegetables

Vegetables were obtained from stalls where a) farmers sell directly to the consumer; and b) market produce was sold. Vegetables were collected twice a month on a Saturday in January and February in 1998; 09/01, 26/01, 09/02 and 23/02 and tests were performed on the corresponding Monday. The vegetables were stored in plastic bags in the fridge for two days until the products were analysed. The vegetable samples investigated were beans (4), broccoli (4), cabbage (7), carrots (8), lettuce (7), mushrooms (7), spinach (5), cucumber (1), celery (1) and tomato (1).

2.2 STANDARD COUNTS

Dilutions were prepared for total, coliform and *E. coli* counts by means of a 10-fold dilution series made in Ringer's solution (Oxoid, Unipath Limited, Basingstoke, Hampshire, England) to 10^{-4} for each sample.

Total counts were determined using Petrifilm™ Total aerobic count –3M (St. Paul, MN, USA). From the 10^{-2} and 10^{-3} dilutions, 1 ml was plated from each dilution onto the petrifilm. When high counts were expected, such as milk from the pasteurised depots, 1ml each of a 10^{-3} and a 10^{-4} dilution was plated. The sample was evenly distributed with the flat side of the spreader provided by the company. The inoculated petrifilm was incubated in the horizontal position (stacks not exceeding 20) at 35°C for 48 h, after which counts were recorded.
Total coliform counts were determined using Petrifilm™ Total coliforms by plating 1 ml of the undiluted sample and 1 ml of a 10^{-1} dilution onto the petrifilm. The sample was evenly distributed and the inoculated petriflim incubated at 35°C for 24 h, after which time counts were recorded.
Total *E. coli* counts were determined using Petrifilm™ Total *E. coli* by plating 1ml of the undiluted sample and 1 ml of a 10^{-1} dilution onto the petrifilm. The sample was evenly distributed and the inoculated petrifilm incubated for 35°C for 24 h, after which time the number of blue colonies with entrapped gas were recorded.
Standards for milk as in the Foodstuffs, Cosmetics and Disinfectants Act, 1972, shown in Table 2.1, were used for interpreting the bacterial counts obtained from the milk samples investigated.

Table 2.1: Summary of Total Microbiologic Counts per millilitre milk
Standards for milk as in the Foodstuffs, Cosmetics and Disinfectants Act. (Act No.54 of 1972). Regulation 1555 of 21 November 1997.

STATE OF MILK	TOTAL VIABLE COUNT	TOTAL COLIFORMS (dry film method)	TOTAL *E. coli*
Raw milk for processing	≤ 200000 cfu/ml	≤20 cfu/ml	<10 colonies/ml
Pasteurised milk	≤ 50000 cfu/ml	≤20 cfu/ml	Absent in 1ml

2.3 ISOLATION OF BACTERIA ON SELECTIVE MEDIA

2.3.1 Sample Preparation and Inoculation Quantities

2.3.1.1 Milk

To concentrate bacteria in milk, 10 ml aliquots were centrifuged for 10 minutes at 4000 x**g**. The skimmed milk was discarded (8 ml) and the fat, sediment and remaining skimmed milk were mixed. Aliquots (50 µl) were streaked on the selective media plates, MacConkey agar and blood agar plates and 500 µl added to enrichment broth, when used.

2.3.1.2 Vegetables

Vegetable samples were prepared in a Waring Blender (Waring Product Division, Dynamics Corporation of America, New Hartfort, Connecticut, USA) with two settings. The blender was initially sterilised in the autoclave at 121°C for 15 min before each batch. After duplicate samples had been treated, the blender was washed, rinsed with 99% ethanol, left with ethanol for 10 min and then inverted in an oven at 80°C for 10 min and cooled before the next sample was mixed. To 90 ml sterile buffered peptone water (Oxoid), 10 g of vegetable sample was added and the mixture was poured into the blender. Mixing of the sample continued until the sample was well suspended (approximately 5 min). The blender was switched continuously on and off to prevent clogging of the sample on the blades. After blending, 50 µl of each sample was spread on selective agars and 500 µl transferred to enrichment broth.

2.3.2 Isolation of Pathogens

Pathogens were isolated and selected from specialised media according to manufacturers' protocols. Flow diagrams have been designed to outline procedures and specific comments added that were taken into consideration during isolation (sections 2.3.2.1-2.3.2.11).

2.3.2.1 Isolation of Staphylococci

Aliquots of milk were streaked onto Baird Parker agar (Oxoid, Unipath Limited, Basingstoke, Hampshire, England) that was enriched with egg yolk tellerite emulsion (Oxoid). Presumptive positive *S. aureus* colonies are black-centred colonies with a clear outer zone and an opaque zone on the agar. Colonies were plated onto blood agar. A gram stain was performed and strains were tested for coagulase production with the Staphytect (Oxoid) test. *S. aureus* is gram-positive cocci in clusters and coagulase positive. Gram positive but coagulase negative colonies were classified as coagulase negative staphylococci (CNS).

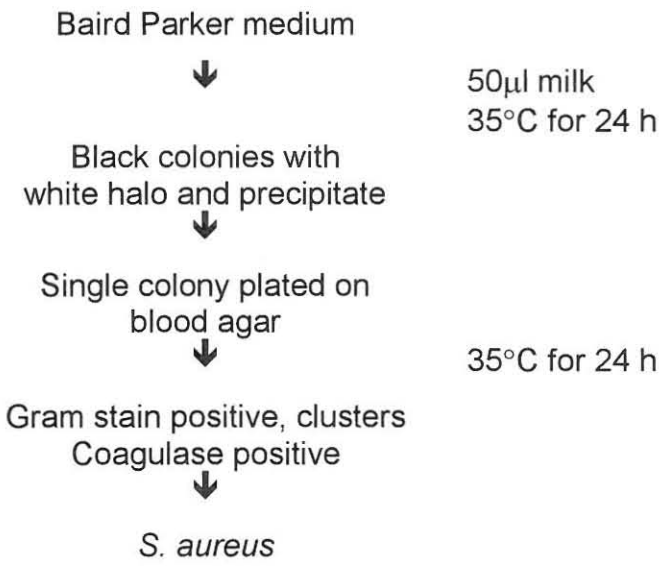

Fig. 2.1 Flow diagram for the isolation of staphylococci

2.3.2.2 Isolation of Streptococci

Edwards medium (Oxoid) was enriched with 5% sheep blood. Blue colonies, taken as presumptive *Streptococcus agalactiae,* were isolated on blood agar. Colonies that showed haemolysis, although weak, were confirmed with the CAMP-test (Collins, Lyne & Grange, 1995). For the CAMP-test a β-haemolytic strain of *S. aureus* was streaked across the centre of a blood agar plate and, at right angles, a presumptive blue colony isolate was inoculated towards the *S. aureus*, without touching it. Five blue colonies were tested per plate and *Enterococcus faecalis* was used as a negative control. The plates are incubated at 35°C for 24 h. A positive *Str. agalactiae* forms a distinct arrow of haemolysis at the junction with the *S. aureus*.

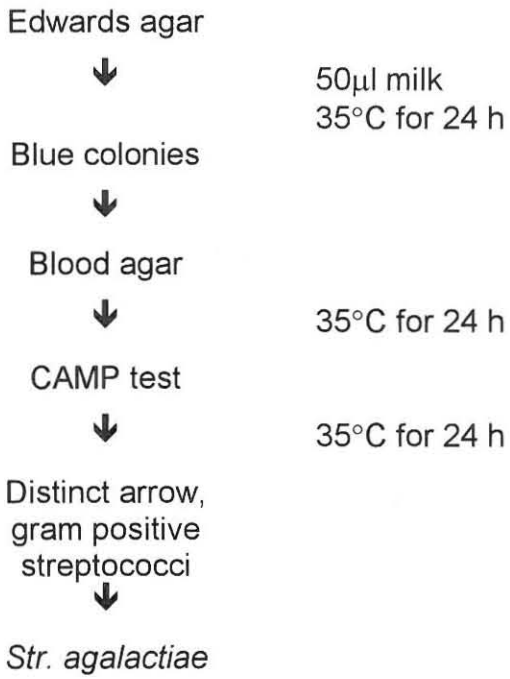

Fig.2.2 Flow diagram for the isolation of streptococci

2.3.2.3 Isolation of Enteropathogenic *E. Coli*

From MacConkey No.3 Agar (Oxoid), three lactose fermenting (LF) colonies typical of *E. coli* were selected per plated sample. The colonies were plated onto blood agar, incubated at 35°C for 24 h and then typed with *E. coli* O Antisera - Monovalent 018 and polyvalent 2 (factors 026, 055, 0111, 0119, 0126), polyvalent 3 (factors 086, 0114, 0125, 0127), polyvalent 4 (factors 044, 0112, 0124, 0142) (Mast Assure stock antisera, Davies Diagnostics, Merseyside, U.K.). Agglutination with any one of these antisera implicates enteropathogenicity in conjunction with API 20E (bioMérieux sa, RCS LYON B, Marcy-l'Etiole, France) identification confirmation. The same colonies were also plated on Sorbitol MacConkey agar (Oxoid) and incubated 35°C for 24 h. *E. coli* 0157 is sorbitol negative with the majority of other *E. coli* strain types being sorbitol positive (Sharp *et al.*, 1994).

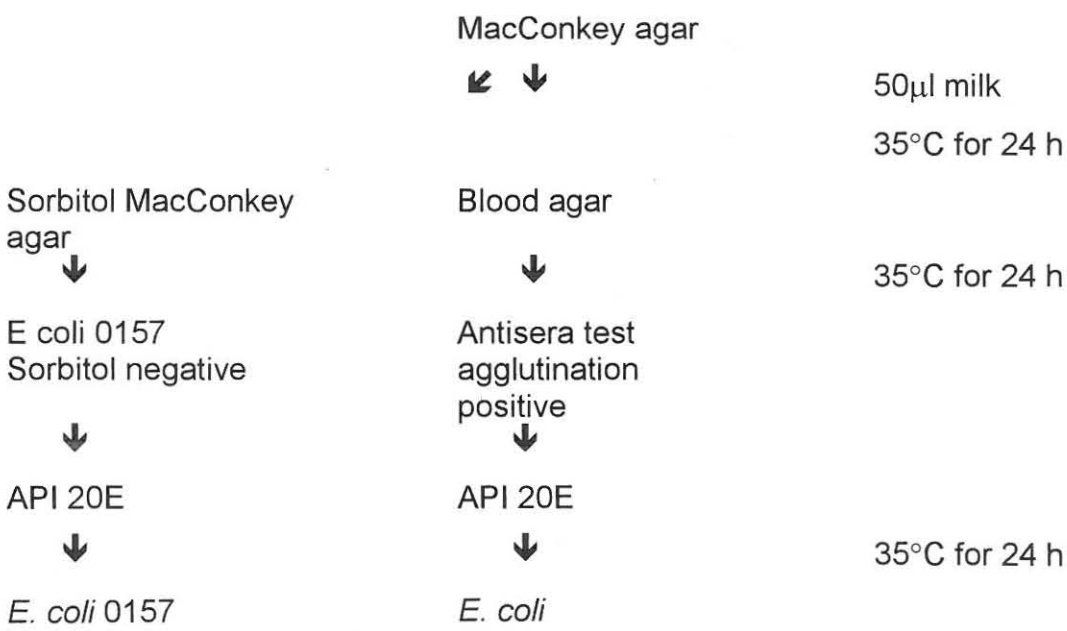

Fig. 2.3 Flow diagram for the isolation of enteropathogenic *E. coli*

2.3.2.4 Isolation of Salmonella and Shigella

Samples were inoculated at a 10^{-1} dilution into selenite enrichment broth (Oxoid). Broth cultures were then transferred to SS agar (Oxoid). *Salmonella* colonies are transparent with a black centre, while *Shigella* colonies are opaque with a brownish centre. Presumptive *Salmonella* colonies were streaked onto Brilliant Green agar (Oxoid). *Salmonella* produce red colonies while *Shigella* species cannot grow. *Citrobacter* species also give red colonies, but they are small. Positive colonies were confirmed with API 20E (bioMérieux).

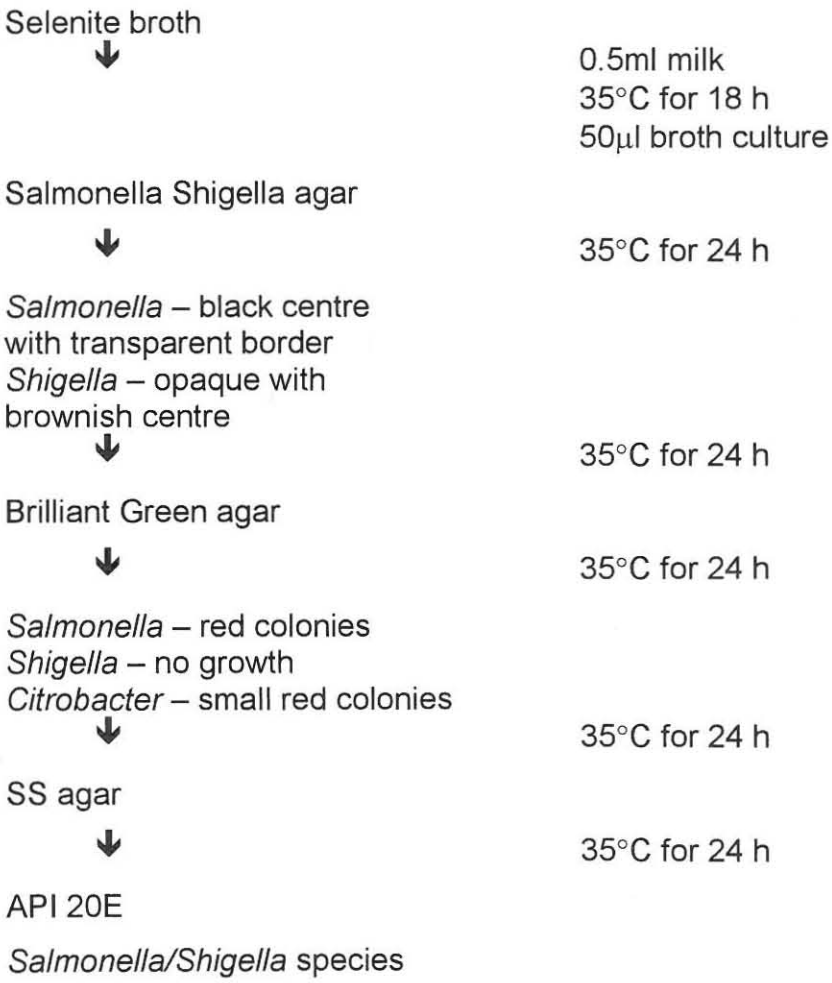

Fig. 2.4 Flow diagram for the isolation of *Salmonella* and *Shigella*

2.3.2.5 Isolation of Yersinia

Yersinia selective agar (Oxoid) was enriched with CIN supplement (Oxoid). A second phase of 24 to 48 h in the fridge is required for slow-growing and psychrotrophic strains. Typical colonies are transparent with a dark red centre, smaller than 1 mm. Presumptive colonies were transferred to Kligler Iron agar (Oxoid) slopes. Kligler Iron agar is a medium based on double sugar fermentation and hydrogen sulphide production. An alkaline slant, acid butt, which means only glucose fermentation has occurred and no gas/sulphur production, indicates a possible *Shigella, Proteus or Yersinia* species. *Yersinia* is a gram negative, ovoid to rod-shaped bacilli confirmed with API 20E (bioMérieux).

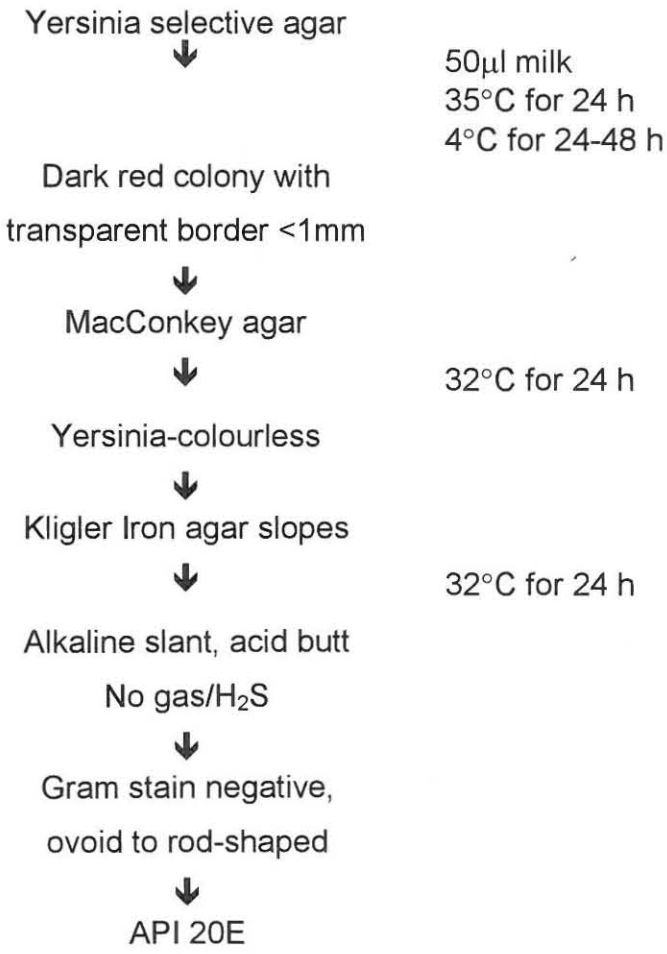

Fig. 2.5 Flow diagram for the isolation of *Yersinia*

2.3.2.6 Isolation of *Campylobacter*

Isolation of campylobacters was performed with the modified CCDA-Preston method as described in the Oxoid Manual. Campylobacter blood-free selective agar base (Oxoid) was enriched with CCDA selective supplement (Oxoid). Plates were incubated for 48 h at 35°C in an anaerobic jar with Campygen (Oxoid) to create a micro-aerophylic condition. Presumptive positive colonies appear grey, moist and spreading and were enumerated on selective agar in a micro aerophylic atmosphere for 48 h at 35°C. *Campylobacter* species are long, thin, gram-negative rods and presumptive colonies were confirmed with the API Campy (bioMérieux).

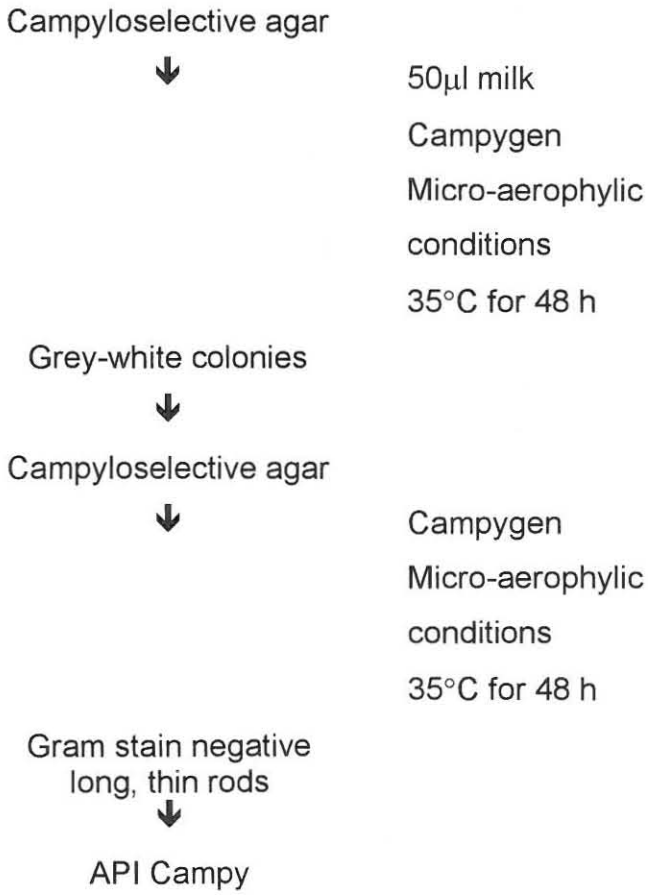

Fig. 2.6 Flow diagram for the isolation of *Campylobacter*

2.3.2.7 Isolation of *Listeria*

A two-stage enrichment procedure was employed to enhance isolation of *Listeria* (Post, 1996). Firstly Listeria enrichment broth (Oxoid), enriched with Listeria selective enrichment supplement (Oxoid), was used, and then Fraser broth (Oxoid) that was enriched with Fraser supplement (Oxoid). From any Fraser medium tube that turned black or showed darkening, broth was plated on Listeria selective agar (Oxoid) enriched with selective supplement (Oxoid). Typical *Listeria* colonies have a black zone around the colony due to aesculin production. Confirmation of colonies was performed with API Listeria (bioMérieux).

Fig. 2.7 Flow diagram for the isolation of *Listeria*

2.3.2.8 Isolation of *Brucella*

Brucella medium base (Oxoid) was enriched with Brucella selective supplement (Oxoid) and 5% inactivated horse serum. The milk was preconditioned by incubation overnight at 40°C. An aliquot of the cream layer was spread on the plates which were incubated in an anaerobic jar with an atmosphere of 10-20% carbon dioxide created by the gas-generating kit (Oxoid) for 10 days. Plates were examined every two days. Typical *Brucella* colonies are white, convex, 1-2 mm diameter, with round entire edges.

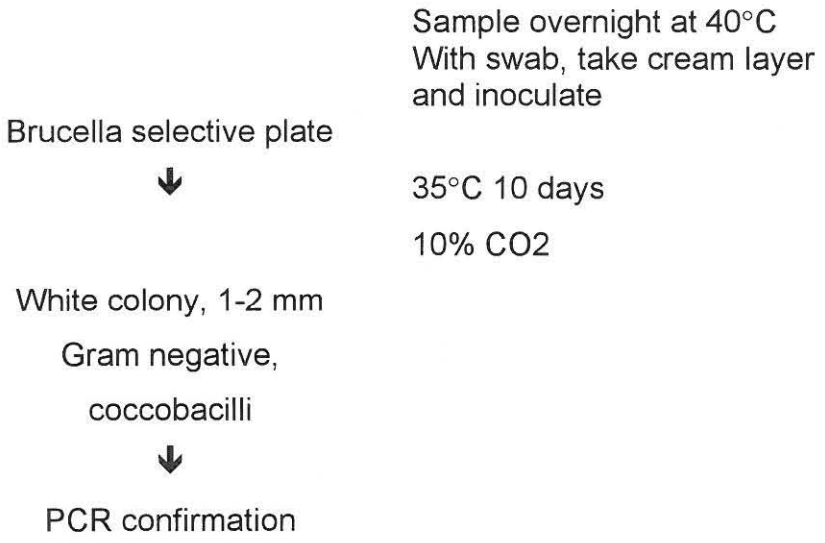

Fig. 2.8 Flow diagram for the isolation of *Brucella*

2.3.2.9 Isolation of *M. bovis*

Isolation of *M. bovis* was performed on Löwenstein and Jenson slopes that were prepared by the Medical Microbiological Laboratory, Universitas Hospital. Slopes were inoculated with 50 µl milk sample and incubated for 6 weeks at 35°C. The slopes were inspected weekly for light, creamy, dry colonies. Presumptive colonies were stained by using the Ziehl Nielsen staining technique, and slides were microscopically viewed for acid-fast bacilli.

2.3.2.10 Isolation of Anaerobes

Two blood agar plates were each inoculated with 50 µl of milk sample. One plate was incubated aerobically and the second in a jar with an anaerobic atmosphere generated by Anaerogen (Oxoid) for 24 h at 35°C. The presence of anaerobic bacteria in milk was determined by comparing the growth of bacteria on the two sets of blood agar plates.

2.3.2.11 Isolation of *B. cereus*

The presence of *B. cereus* was only investigated in vegetables. *Bacillus* cereus selective agar base (Oxoid) enriched with Polymyxin B (Oxoid) was inoculated with 50 µl of homogenised sample. Plates were incubated at 35°C for 24 h and examined for typical colonies with a diameter of 5 mm that had a distinctive turquoise to peacock-blue colour and were crenated.

2.4 NON-MICROBIOLOGICAL TESTS APPLIED TO ASSESS MILK QUALITY

2.4.1 Phosphatase Test

The Aschaffenburg and Mullen phosphatase test gives an indication as to whether a milk sample has been pasteurised successfully or has been contaminated with raw milk after pasteurisation (R. 1555, Government Gazette, 1997). Bacteria produce phosphatase and its presence in pasteurised milk can be indicative of a high bacterial count.

To 100 ml of buffer [0.3% (w/v) anhydrous sodium carbonate + 0.15% (w/v) sodium bicarbonate], 150 mg of disodium p-nitrophenyl phosphate was added and dissolved. The solution should be kept in a dark bottle in the fridge and made up freshly every two to three days. To 5 ml of the phosphate buffer, 1 ml of milk was added in a sterile test tube. After mixing the solution, the tubes were incubated for 2 h at 37°C in a water bath. The colour of the milk samples was

compared in a Lovibond comparator with a APTW 5 disc. Distilled water was used as a control and should give a value of less than 10 μg p-nitrophenol. A value greater than 10 μg p-nitrophenol showed that the sample was either not well-pasteurised or had been contaminated with raw milk after pasteurisation.

2.4.2 Detection of Inhibitory Substances

Kundrat Medium (Merck KGaA, Darmstadt, Germany) is an agar base that is inoculated with a *Bacillus stearothermophilis* spore suspension (Merck). The cooled molten agar containing the spores is poured into a petri dish and allowed to solidify.

An aliquot of milk was placed in a water bath at 80°C for 10 min, after which a Whatman filter disk AA 0.5 (Merck) was impregnated with a 100 μl sample. The disk was then placed onto the agar containing the lawn of spores and incubated for 2-3 h. A clear zone around disk was an indication of a possible inhibitory substance (Cullor, 1993).

2.4.3 Stability Test

The stability of the milk was measured by mixing equal parts of 68% (v/v) ethanol with milk (2 ml milk and 2 ml 68% ethanol) (R. 1555, Government Gazette, 1997). Signs of coagulation can be an indication of high somatic cell counts or high bacterial counts.

2.5 PCR DECTECTION AND BACTERIAL CONFIRMATION TECHNIQUES

2.5.1 Preparation of Target DNA

2.5.1.1 Milk

Direct analysis: Milk analysed directly was heated to 99°C for 10 min in a Perkin Elmer PCR machine.

Dynabead: The **DYNAL** Dynabeads® DNA DIRECT™ system 1 (DYNAL A.S, Skøyen, Oslo, Norway) is used for the isolation and concentration of PCR-ready genomic DNA.

The Dynabead procedure as described by the manufacturer was followed:

1. Bacterial cells were pelleted from 200 µl of milk by centrifugation at 5000 x**g** for 5 min and the supernatant discarded.
2. The sample size was 200 µl and 200 µl of dynabeads was added in single pipetting action.
3. Lysis was performed at 65°C for 15 min. The microcentifuge tube was then placed in the Dynal MPC unit and the supernatant removed without disturbing the complex.
4. The microcentrifuge tube was then removed from the Dynal MPC unit and the dynabead complex washed with 200 µl washing buffer.
5. The tube was then replaced in the Dynal MPC unit and the supernatant removed. Washing was repeated once more and on the final removal of the washing buffer, no residual buffer was present.
6. Tubes were then removed from the Dynal MPC and the DNA/dynabead complex resuspended in 20 µl resuspension buffer. The complex was carefully pipetted up and down until the suspension was homogeneous.
7. The DNA was eluated by incubation at 65°C for 5 min.
8. Target DNA was held at –70°C until required. Elution at 65°C for 5 min was performed just prior to addition to the PCR mix.

2.5.1.2 Bacterial Cultures

Lysis: Target DNA was prepared by lysing four colonies in 2.5 ml Tris/EDTA buffer [pH 6.8] with lysozyme 12.5 µg for 20 min at 25°C, followed by the addition of 1% Triton-X (v/v) and proteinase K 6.25 µg and incubation at 37°C for 1-2 h.

Dynabead: Dynabeads were also employed for preparing DNA from bacterial cultures to ensure the system was working satisfactory. A suspension of bacterial cells (approximately 10^7 cfu/ml) was made in Ringers solution. Cells were centrifuged at 7000x**g** for 5 min and the supernatant removed. To the pellet, 200 µl of dynabeads was added and the DNA extracted as described above in section 2.5.1.1.

2.5.2 PCR Technique

Amplification was performed in a total volume of 25 µl; the reaction mix comprising 1.25 µl of target DNA, 10 mM Tris-HCl [pH 8.3]; 2.5 mM $MgCl_2$, 50 mM KCl; 200 µM of each dNTP and 25 pmols of each specific primer (Table 2.2). Amplification: 1 cycle of 5 min at 94°C cooling to the required annealing temperature (Table 2.2) at which temperature 0.25 units of *taq* DNA polymerase (Expand High Fidelity, Boehringer Mannheim, Mannheim, Germany) was added, followed by 5 min at 72°C. The number of cycles as recommended by the individual research groups (Table 2.2), were then performed. On completion of the cycling programme, an additional final extension time of 5 min was included. PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels and the 100 bp molecular weight standard XIV (Boehringer Mannheim) was used as a size marker in each gel run.

Table 2.2 Details of primers and corresponding PCR conditions

Species	Primer target	Sequence	Temperatures and times	No. of cycles	Fragment size (bp)	Reference
All Universal primers	Conserved domain 16S rRNA	U1 5'-CAGCAGCCGCGGTAATCC U2 5'-CCGTCAATTCATTTGAGTTT	95°C; 1 min 50°C; 1 min 72°C; 1 min	30	408	Siggens (1995)
L. monocytogenes	Listeriolysin O gene	LM1 5'-CCTAAGACGCCAATCGAA LM2 5'-AAGCGCTTGCAACTGCTC	95°C; 1min 50°C; 1 min 72°C; 1min	30	702	Siggens (1995)
C. jejuni	Open reading frame	CJ1 5'-AGAACACGCGGACCTATATA CJ2 5'-CGATGCATCCAGGTAATGTAT	95°C; 1min 60°C; 1 min 72°C; 1 min	35	256	Jackson, Fox & Jones (1996)
C. jejuni	23S rDNA	CJ3 5'-TAAAGTAAGTACCGAAGCTG CJ4 5'-GTAAATCCTAATACAAAGCT	95°C; 1min 54°C; 1min 72°C; 1min	35	710	Eyers et al (1993)
B. abortus	IS*711* and flanking region	B1 5'-GACGAACGGAATTTTTCCAATCCC B2 5'-TGCCGATCACTTAAGGGCCTTCAT	95°C; 1min 54°C; 1.5 min 72°C; 2min	30	498	Bricker & Halling (1994)
B. abortus	16S rRNA	B3 5'-TCGAGCGCCCGCAAGGGG B4 5'-AACCATAGTGTCTCCACTAA	95°C; 1min 54°C; 1.5 min 72°C; 2 min	30	905	Romero et al (1995)

2.6 DETECTION OF ANTIBIOTIC-RESISTANT BACTERIA

2.6.1 Media and Antibiotics

Media used to prepare plates for screening were MacConkey agar (Oxoid) and Mueller-Hinton agar (Oxoid).
Media used for preparing culture dilutions were Mueller-Hinton broth (Oxoid) and for breakpoint screening/MICs, Mueller-Hinton agar (Oxoid).

Standard antibiotic powders with stated potencies were obtained from Sigma (St Louis, USA) as detailed in Table 2.3. Also given, are respective resistance breakpoints determined by the National Committee for Clinical Laboratory Standards [NCCLS] (1998).

Table 2.3 Details of antibiotic powders

Antibiotic	Abbrev.	Potency	Resistance breakpoint(µg/ml)
Penicillin G	PEN	1661 units/mg	0.25
Ampicillin	AMPC	98%	16
Cefoxitin	CFOX	99.4%	16
Cefotaxime	CTAX	95%	32
Ceftriaxone	CTRX	100%	32
Erythromycin	ERY	98%	1
Tetracycline	TET	95%	8
Rifampicin	RIF	95%	2
Streptomycin	STR	765 units/mg	8
Gentamicin	GEN	647 µg/mg	8
Kanamycin	KAN	784 µg/mg	32
Vancomycin	VAN	1099 units/mg	8
Chloramphenicol	CHL	100%	16

As some antibiotic powders were either not available or too expensive to include in a screening programme, antibiotic disks were obtained from Mast Laboratories (Merseyside, UK) concentrations and resistant zone sizes (NCCLS, 1998) are given in Table 2.4.

Table 2.4 Details of antibiotic disks

Antibiotic	Abbrev.	Disk conc. (µg)	Resistant zone size (mm)
Augmentin*	AMOX/CA	20	≤13
Imipenem	IMI	10	≤13
Cotrimoxazole†	SMX/TMP	25	≤10
Ciprofloxacin	CIPX	5	≤15
Oxacillin	OXAC	1	≤10

* Augmentin is a combination of amoxycillin and clavulanic acid.

† Cotrimoxazole is a combination of sulphamethoxazole and trimethoprim.

2.6.2 Screening for Antibiotic-resistant Bacteria

Initial screening of milk samples was performed by incorporating antibiotic dilutions into MacConkey agar from which gram-negative bacteria and enterococci were enumerated, and Mueller-Hinton for the presence of gram positive bacteria.

Final antibiotic concentrations for the selection of gram-negative bacteria were: ampicillin16 µg/ml, cefoxitin 16 µg/ml, cefotaxime 16 µg/ml, kanamycin 16 µg/ml, streptomycin 8 µg/ml, gentamicin 8 µg/ml, tetracycline 8 µg/ml and chloramphenicol 16 µg/ml.

Final antibiotic concentrations for selection of gram-positive bacteria were: penicillin 8.0 and 0.125 µg/ml, cefoxitin 16 µg/ml, vancomycin 16 and 2 µg/ml, gentamicin 8 µg/ml, tetracycline 8 µg/ml, erythromycin 4 µg/ml, rifampicin 2 µg/ml and chloramphenicol 16 µg/ml.

Milk (100 µl) was spread on the surface of each plate in the antibiotic batch series. Inoculated plates were incubated at 37°C for 24 h, after which colonies were examined, enumerated and selected for confirmatory screening.

2.6.3 Confirmatory Screening of Individual Strains for Antibiotic Resistance

Bacterial strains collected directly from milk, from antibiotic screening plates and from vegetable cultures were grown on Mueller-Hinton agar plates for 18-24 h at 37°C. A suspension of cells ($\leq 10^7$ cfu/ml) was made in Mueller-Hinton broth and the inoculated broth was incubated until turbid at 35°C for 2-3 h. The cultures were then standardised to a McFarland 0.5 in Mueller-Hinton broth diluted 1/10 and inoculated onto plates containing antibiotic concentrations by means of a multipoint inoculator (Mast Laboratories), final cell concentration per spot 10^4 cfu/ml. Plates were incubated at 35°C for 18 h and inoculated areas recorded as growth or no growth. Antibiotics were incorporated into plates at their respective resistance breakpoint concentrations (Table 2.3).

For gram-negative bacteria the following was used: ampicillin16 µg/ml, cefoxitin 16 µg/ml, cefotaxime 32 µg/ml, ceftriaxone 32 µg/ml, kanamycin 32 µg/ml, streptomycin 8 µg/ml, gentamicin 8 µg/ml, tetracycline 8 µg/ml and chloramphenicol 16 µg/ml.

For gram-positive bacteria the following was used: penicillin 8.0 and 0.125 µg/ml, vancomycin 16 and 2 µg/ml, gentamicin 8 µg/ml, streptomycin 8 µg/ml, tetracycline 8 µg/ml, erythromycin 4 µg/ml, rifampicin 2 µg/ml and chloramphenicol 16 µg/ml.

Control cultures were included in each antibiotic batch series, were *S. aureus* ATCC 29213 and *E. coli* ATCC 25922.

2.6.4 Minimum Inhibitory Concentration (MIC) Determinations

Minimum inhibitory concentrations were determined by the agar dilution method as described by the NCCLS (1997a). Logarithmic growth phase cultures were prepared and inoculated onto antibiotic plates as described in section 2.6.3. Antibiotics were incorporated into Mueller-Hinton agar to form a 2-fold dilution

series, range 0.06-128 µg/ml. Plates were incubated at 35°C for 18 h. MIC was defined as the lowest concentration of antibiotic which inhibited (≤ 5 colonies per spot) the bacteria.

Control cultures included in each series, were *S. aureus* ATCC 29213 and *E. coli* ATCC 25922.

2.6.5 Disk Susceptibility Tests

Disk susceptibility tests were performed as described by the NCCLS (1997b). The inoculum was prepared by transferring 3-5 isolated colonies to Mueller-Hinton broth and the culture incubated at 35°C until a turbidity equivalent to a 0.5 McFarland standard was reached (± 2 h, approximately $1\text{-}2x10^{-8}$ cfu/ml). A sterile cotton swab was dipped into the cell suspension, excess inoculum removed from the swab, which was then streaked over the entire surface of a dried Mueller-Hinton agar plate. Disks were placed on the inoculated agar plates (not more than 5 disks/100 mm plate). Within 15 min of applying the disks the plates were inverted and placed in an incubator at 35°C for 16-18 h. Zones of inhibition were measured to the nearest mm with calipers and resistance determined according to the NCCLS (1998) resistant zone sizes given in Table 2.4.

CHAPTER 3

MICROBIOLOGICAL STATUS OF MILK AND VEGETABLES

3.1 INTRODUCTION

Contamination of milk can occur by numerous routes with different bacteria of varying degrees of pathogenicity at all stages of collection and processing. The concept that microbiological limits be introduced to ensure safety and overall quality of foods was accepted early in the 1900s. Mandatory criteria have been established to contain limits of pathogens of public health significance and to set standards for non-pathogens. In South Africa, legislation requires the microbiological status of milk to conform to standards as described in an amendment to the Foodstuffs, Cosmetics and Disinfectants Act of 1972 (Table 3.1). The counts for the total coliforms are determined by using the dry film method.

Table 3.1 Standards for milk

Foodstuffs, Cosmetics and Disinfectants Act,
(Act No.54 of 1972). Regulation 1555 of 21 November 1997.

MILK TYPE	TOTAL COUNT	COLIFORMS	*E. COLI*
Raw milk for processing	≤200000 cfu/ml	≤20 cfu/ml	<10 cfu/ml
Pasteurised milk	≤50000 cfu/ml	≤20 cfu/ml	Absent in 1 ml

In the USA, raw milk batches for pasteurisation may contain ≤50000 bacteria/ml (total count) and, after pasteurisation, ≤15000 bacteria/ml, coliform count ≤10/ml to be graded as Grade A milk (Jay, 1992). Standards for pasteurised milk according to the English legislation by Statutory Instrument 1989 No. 2383 are for raw milk total count ≤50000/ml, coliform count ≤5/ml and for pasteurised milk total count ≤30000/ml, coliform count ≤1/ml (Burden *et al.*, 1995; Maura, Destro & Franco, 1993; Ombui *et al.*, 1994). The South African standards for milk

(Table 3.1) are less strict than those of the USA and England. It is important to have a standard for a total bacterial count applicable to raw milk as the larger the number of bacteria, the higher the degree of heat resistance which could seriously compromise pasteurisation processes (Jay, 1992).

In many countries milk standards are based on the enumeration of non-fastidious bacteria ("indicators of food safety") and excludes, with the exception of *E. coli,* a full list of pathogens that should be excluded from milk and milk products. Many different selective and enrichment media and identification systems have been introduced to isolate and confirm the identity of pathogens that may be present in milk (Chapter 1), but directives are lacking as to how to perform such tests. A major problem with the microbiological screening for pathogens is that bacterial cells may be in a state of metabolic injury (e.g. sub-lethal heat injury), resulting in their inability to form colonies on selective media that uninjured cells can tolerate (Jay, 1992). Such metabolically injured cells may escape detection. However, recovery may occur and once fully viable, their infection potential is re-realised.

The phosphatase test is used as an indicator of successful pasteurisation as phosphate is inactivated by heat (Early, 1992). Detecting the presence of inhibitory substances, especially antibiotics, are of importance in providing milk of a good quality (Crosby, 1991; Cullor, 1993). The alcohol stability test provides an indication as to whether the milk will remain stable on further processing, especially with heat treatment. Any signs of coagulation might be an indication of colostrum or a high somatic cell count (Early, 1992).

Safety standards on the microbiological content of raw vegetables are problematic as they often fail due to inherently high numbers of coliform bacteria (Jay, 1992). Contamination of vegetables with pathogens is a direct consequence of agricultural practices, in particular the use of animal/human manure, irrigation with contaminated water and contamination caused by food handlers (Varnam & Evans, 1991). Hazards are obviously greater when vegetables are eaten raw. Controls over storage to protect the shelf life of vegetables are difficult to impose with post-harvest storage in refrigerators

actually increasing the development of low-temperature pathogens such as *Listeria*.

3.2 RESULTS AND DISCUSSION

3.2.1 Non-microbiological Status

The phosphatase test was applied to the milk samples to give an indication as to whether the milk was, as described, raw or pasteurised (Appendix, Table A1). Raw milks (Table 3.2, milks 13 and 38) were actually sold to the public from a depot and the milk did not comply with the raw milk standards. Two samples from depots (Table 3.2, samples 34 and 35) were found not satisfactorily pasteurised with both milks failing microbiologically on coliform counts and one on *E. coli* content. Two raw batch samples failed the stability test. No inhibitory substances such as antibiotic residues were detected. However, it has been proposed that more sophisticated methods should be applied, other than the use of the standard technique, employing a sensitive bacteria, as the concentration of inhibitory substances may be present at parts per billion (10^{-9}) or lower (Crosby, 1991).

3.2.2 Enumeration of Bacteria Present in Milk

Petrifilms were used to enumerate total non-fastidious bacteria, coliform and the *E. coli* content of the milk sampled. The respective counts for the milks sampled over the 14-month period from February 1997 to March 1998 are shown in Tables 3.2 and 3.3. It can be seen that samples from individual cows and raw batches were of a good quality with regard to total counts. Raw milk for processing with relatively low counts (although higher than specified) was seen to meet post-pasteurisation standards (Table 3.2, milks 27 and 28).

Table 3.2 Milk samples collected in 1997

Lab. No.	Origin	Producer	Counts (cfu/ml) Total	Coliforms	*E.coli*
	SUMMER				
	FEBRUARY-MARCH				
9	**COW:**	A	23000	10	0
10	raw	A	36000	100 *	0
11		A	17000	<10	0
15		B	1000	30 *	0
16		B	7000	150 *	220 *
17		B	2100	<10	0
12	**BATCH:**	A	4000	40 *	1
13	raw	C	390000 *	400 *	120 *
18		B	60000	>20000 *	0
5	**DEPOT:**	D	42000	50 *	10 *
6	pasteurised	E	137000 *	>3000 *	90 *
7		E	88000 *	>3000 *	80 *
8		F	5200	170 *	0
14		A	460000 *	6600 *	160 *
1	**INDUSTRY:**	G	400	<10	0
2	pasteurised	H	124000 *	60 *	0
3		I	700	120 *	0
4		J	22000	160 *	0
	AUTUMN				
	APRIL				
19	**COW:**	F	45000	10	4
20	raw	F	7000	10	0
21		F	150000	>900 *	0
24		D	16500	260 *	34
25		D	15000	540 *	26
26		D	30000	300 *	18

Table 3.2 continued

27	**BATCH:**	D	29000	750 *	29
29	raw	G	181000	600 *	8
22	**BATCH:**	F	800	<10	0
23	pasteurised	F	220000 *	>3000 *	0
28		D	2400	<10	0
	WINTER				
	MAY				
30	**BATCH:**	K	106000	470 *	200 *
31	raw	L	8000	520 *	7
32		M	105000	280 *	34
33	**BATCH:**	N	9000	100 *	1 *
	pasteurised				
	SPRING				
	OCTOBER				
38	**BATCH:**	C	300000 *	4500 *	190 *
39	raw	P	3000	<10	0
41		Q	600000 *	4200 *	50
42		K	3000	10	10
43		R	700000 *	6000 *	60
44		S	14000	2000 *	2
34	**DEPOT:**	E	10000	200 *	2 *
35	pasteurised	E	3000	180 *	0
36		A	700000 *	>3000 *	0
37		F	120000 *	>3000 *	0
40		A	260000 *	>3000 *	10 *
45		N	3000	<10	1 *

* Counts that did not conform to standards

A-S = Different producers: Individual cows and raw milk batches collected at farms and selling points in town; depots raw and depots and industries pasteurised.

Table 3.3 Milk samples collected in 1998

Lab. No.	Origin	Produce	Counts (cfu/ml)		
			Total	Coliforms	*E.coli*
	AUTUMN				
	END MARCH				
46	**BATCH:**	P	9000	100 *	0
47	raw	A	40000	150 *	80 *
48		D	2100	20	0
49		F	15000	50 *	6
53		R	200000	9000 *	30 *
55		S	44000	320 *	0
50	**DEPOT:**	F	13000	250 *	0
51	pasteurised	E	180000 *	>10000 *	0
52		C	70000 *	1800 *	20 *
54		N	2200	0	0

* Counts that did not conform to standards

A-S = Different producers: Individual cows and raw milk batches collected at farms and selling points in town; depots raw and depots/industries pasteurised.

On comparing milk samples from producers A and F from individual cows to raw batches to the depot product, there was an increasing tendency for counts to exceed standards. An overall summary of the microbiological status of the milk investigated is shown in Table 3.4. Of a total of 20 raw batch samples tested, 85% did not conform to standards for raw milk and 19 of the 32 (82.6%) of depot and industrially pasteurised milks did not conform. Cause for concern was that five of the nineteen depot milks exceeded all three standards.

Table 3.4 Summary of milk samples that did not conform to standards

Type of milk	Season	No. of samples	No. not conforming	Tests that did not conform				
				Coli	Ec	TC, Coli	Coli, Ec	TC, Coli Ec
COWS	Summer 1997	6	3	2			1	
	Autumn 1997	6	4				4	
	TOTAL	**12**	**7**	**2**			**5**	
RAW	Summer 1997	3	3	2				1
	Autumn 1997	2	2	2				
	Winter 1997	3	3	2			1	
	Spring 1997	6	4	1		2		1
	Autumn 1998	6	5	5				
	TOTAL	**20**	**17**	**12**		**2**	**1**	**2**
DEPOTS	Summer 1997	5	5	1			1	3
	Autumn 1997	3	1			1		
	Winter 1997	1	1	1				
	Spring 1997	6	6	1	1	2	1	1
	Autumn 1998	4	3	1		1		1
	TOTAL	**19**	**16**	**4**	**1**	**4**	**2**	**5**
INDUSTRIAL	Summer 1997	**4**	**3**			**1**	**2**	
	FINAL TOTAL	**55**	**43**	**18**	**1**	**7**	**10**	**7**

Coli = Coliforms, Ec = *E. coli*, TC = Total counts

The microbiological status of milks from all categories collected from the Bloemfontein area during 1997 and 1998 were generally considered unsatisfactory. The latest South African regulation has stricter standards for the presence of coliform bacteria in raw milk due to the selling of raw milk to the

public, but this is ludicrous and short-sighted. Raw milk should not be consumed at all. Other pathogens than the *Enterobacteriacea,* are not in the regulation control programme.

Raw milk tested in Ireland in 1992 (13-month period, 589 milks) gives an indication as to first-world farming success rate, with 63% of samples having total bacterial counts of <30000 cfu/ml, 65% <100 coliforms/ml and 60% ≤10 *E. coli*/ml (Rea, Cogan & Tobin, 1992). In a study conducted by the public health laboratory at the Royal Berkshire Hospital, UK in 1994, 2690 pasteurised milk samples were tested. Only 96 (3.6%) of the samples had total counts that exceeded 30000cfu/ml and 22.6% had more than one coliform/ml (Burden *et al.,* 1995). Burden *et al.* (1995) also found that only 3.6% of milk samples from small dairies exceeded British standards concerning total counts, but there was a significantly higher coliform count and 1.2% of milk was found to test positive for phosphatase. In Nairobi, Kenya, it was found in 1994, that 89.5% of milk from farmers and 50% of milk from co-operative industries had less than 50000 cfu/ml and 42.2% of farmers and 10.3% of co-operative industries had no coliforms/ml (Ombui *et al.,* 1994). When compared to another African country, the Kenyan study showed that the milk quality obtained from Bloemfontein compare to third world standards.

3.2.3 Detection of Pathogenic Bacteria in Milk

Enrichment culture media was employed for *Salmonella* and *Listeria* detection. The supplements contain a variety of enriching nutrients that are capable of supporting/promoting growth of desired bacteria or assist in the recovery of heat-stressed/injured cells that may be present in small numbers (Cappuccino & Sherman, 1996). Selective media was then used to isolate potentially pathogenic bacteria. Presumptive colonies were selected according to the manufacturers' protocol pertaining to the different selective media employed. Confirmation of a bacterial species was generally performed by Gram stain and using the appropriate API identification system.

It can be seen in the present study that colony morphology and in-agar biochemical testing is not satisfactory (Table 3.5). In a study comparing three new chromogenic agar mediums for *E. coli* detection, the results obtained were in accordance to those achieved by conventional methods, but in some cases the appearance of colonies were not completely in accordance with the manufacturers' indications (Grand & Baumgartner, 1996). Wallace & Jones (1996), found that of 614 presumptive *E. coli* 0157 strains, obtained from the faeces of dairy herds, that were isolated on selective chroma agar 0157, 99% were false positives. Even after isolation, problems are evident in obtaining definite identifications to species level, especially in the case of environmental strains. An example of this was reported by Gilmor & Harvey (1990) with regard to the enumeration of *S. aureus* from dairy products. In that study some strains were not as black as typical colonies and some did not give an egg yolk reaction. Positive coagulase reaction is the most widely accepted means of confirming suspected colonies of *S. aureus,* but *S. chromogenes* and *S. hyicus* also produce coagulase (Gilmour & Harvey, 1990).

In the present study, identification of the majority of presumptive pathogens was performed using API systems. To perform API tests, pure cultures are necessary and this requires one to three subcultures with a growth period of 18 to 24 h for each stage of purification. Certain precautions must be adhered to concerning the API systems. It is necessary to select a trained and experienced candidate to predetermine gram stain reaction and/or microscopy in order to employ the correct system. The data bank for interpreting API profiles is based on clinical isolates and it might well be expected that bacteria from environmental sources may not totally conform.

Table 3.5 Presumptive pathogenic bacteria from milk

Selective agar	Presumptive pathogen	No. of strains	No. incorrect ID	Identified strains
Listeria	*Listeria*	4	0	*L. monocytogenes*
Campylobacter	*Campylobacter*	7	7	Not *Campylobacter*
Edwards	*Str. agalactiae*	21	21	*Enterococcus* spp
Yersinia	*Y. enterolitica*	36	36	*Providencia* spp *Pseudomonas* spp *Rahnella aquatilis*
SS	*Salmonella* *Shigella*	11	11	*C. freundii* *Proteus vulgaris* *Hafnia alvei*
MacConkey	EPEC	41	40	***E. coli*** *E. vulneris* *E. hermannii* *Ent. agglomerans* *Lecl. adecarboxylata* *Aer. hydrophila* *C. freundii*
Baird Parker	*S. aureus*	82	42	CNS

GENUS: *Str.=Steptococcus; Y.=Yersinia; L.=Listeria; C.=Citrobacter; E.=Escherichia; Ent.=Enterobacter; Lecl.= Leclercia; Aer.=Aeromonas.*

CNS=Coagulase-negative staphylococci.

**E. coli* correctly selected but not EPEC.

Not one of the colonies from the campyolobacter selective agar was found to be a *Campylobacter* species (Table 3.5). The two-step enrichment and selective media for Listeria, however, performed exceptionally well. API profiling confirmed the identity of presumptive *Listeria* from three raw milks (profiles: 6510, 6010 and

6410) and a pasteurised milk (profile: 6510), all being *L. monocytogenes* (Table 3.5). The incidence of *L. monocytogens* in the raw milk was 3/20 and in pasteurised milk 1/19. It should be noted that the pasteurised milk was purchased from a depot as for consumption.

Listeria was detected in 4.9% of pasteurised milk (2690 samples) in the UK during 1994-1995 of which 0.1% was identified as *L. monocytogenes* (Burden *et al.,* 1995). In Denmark, *Listeria monocytogenes* showed a low but constant level (0.01-0.1%) of infection in individual cows (Jensen *et al.,* 1996). Of raw milk samples examined in Brazil, 12.7% were positive for *Listeria* species and 9.5% were positive for *L. monocytogenes* (Mauro, Destro & Franco, 1993).

ß-Haemolytic streptococci were isolated from two raw milk samples. The one milk sample was obtained from a depot that sold raw milk directly to the public. Group A haemolytic streptococci not only cause mastitis, but are human pathogens. Before wide-scale pasteurisation was implemented, *Streptococcus pyogenes* from clinical mastitis was a common cause of human infection, pharyngitis, and sepsis of lesions, and even complications such as scarlet fever and glomerulonephritis (Varnam & Evans, 1992). An outbreak of ß-haemolytic streptococcal infection in the late 1970s in New Mexico was caused by raw milk which was identified as the vehicle of infection, and two deaths resulted from 16 known cases (Varnam & Evans, 1992).

The relevance of staphylococci in milk is not clearly defined legally, although the presence of *S. aureus* and its enterotoxins in raw milk and inadequate pasteurised milk is well-documented (Gilmour & Harvey, 1990; Ombui, Arimi & Kayihura, 1992). In the present study, 2 (10.5%) of the pasteurised milk samples, and 6 (19%) of the raw milk samples tested positive for S. aureus (Appendix, Table 3A). Overall, CNS were detected in 14/55 milks.

In Dandora, Kenya, 183/300 (61%) raw milk samples at a co-operative dairy tested positive for *S. aureus,* and of these strains, 74.2% were found to produce enterotoxins. Makaya, Aarestrup & Olsen (1996) found that of milk from large-scale commercial dairies, small-scale commercial dairies and communal dairies in Zimbabwe, 10.9% of milk samples contained *S. aureus* and 4.1%, CNS. The

results obtained in the present study showed that the incidence of *S. aureus* in pasteurised milk was similar to that in Zimbabwe, but in raw milk, *S. aureus* and CNS were higher (37.5%) in Bloemfontein, illustrating the importance of pasteurisation. There is increasing evidence that CNS are pathogenic to both animals and humans (Breckenridge & Bergdoll, 1977; Gilmour & Harvey, 1990). A coagulase-negative variant of *S. aureus* was seen to cause mastitis and was considered to be of clinical importance (Breckenridge & Bergdoll, 1977). Foodborne gastroenteritis due to CNS has been reported (Fox, Besser & Jackson, 1996).

3.2.4 Detection of Pathogenic Bacteria in Vegetables

The isolation of pathogenic *Enterobacteriaceae* from vegetables is not an easy task. As vegetables contain high numbers of non-pathogenic strains, it is necessary to spend considerable effort in selecting and confirming the identification of many individual isolates. Many potentially promising pathogens were eventually identified as opportunistic pathogens from many diverse species (Table 3.6). The majority of shigella-like colonies tended to be *Enterobacter sakazakii* and the yersinia-like colonies, *Ent. agglomerans*.

One should not be complacent concerning the non-pathogenic strains from the milk and vegetables identified in Tables 3.5 and 3.6. They are capable of causing a number of infections in the human, especially in cases where the hosts are immuno-compromised. *Citrobacter freundii* is naturally found in soil, but can cause urinary tract infections. *C. freundii* was implicated in a severe outbreak of gastroenteritis in Germany due to parsley which had been fertilised with pig manure (Tschäpe *et al.*, 1995). *Klebsiella pneumoniae, K. aerogenes, K. oxytoca* and *K. ozoenae* have been isolated from respiratory infections (Collins, Lynne & Grange, 1995). Cases have been reported where *Serratia marcescens* caused meningitis, endocarditis and urinary tract infections (Collins, Lynne & Grange, 1995). *Providencia rettgeri* is an opportunistic human pathogen and it is associated with urinary tract infections, while *P. stuartii* has been implicated in hospital infections (Collins, Lynne & Grange, 1995).

Table 3.6 Presumptive gram negative pathogenic bacteria from vegetables

Vegetable		SELECTIVE MEDIA		
no	sample	Yersinia	MacConkey	SS agar
3	Carrots 1	*Serratia*	*Aer. hyrophila*	*Prov. rettgeri*
4	Carrots 2	*Erwinia*		*Ent. sakazakii*
16	Carrots 1			*Ent. sakazakii*
17	Carrots 2		*K. pneumoniae*	
30	Carrots 2	*Ent. agglomerans*		
5	Lettuce 1			*Prov. rettgeri*
14	Lettuce 1	*Kluyvera*		*Ent. sakazakii*
24	Lettuce 2	*Serratia*		
12	Broccoli 2			*C. freundii*
18	Broccoli 2	*Lecl. adecarboxylata*		
13	Cabbage 1	*Ent. agglomerans*		
22	Cabbage 2	*Ent. agglomerans*		
10	Spinach 1		*Aer. hyrophila*	
15	Beans 1	*E. vulneris*	*E. vulneris*	*Ent. sakazakii*
19	Mushrooms 2		*Kluyvera*	
20	Celery 2	*Ent. agglomerans*	*C. freundii*	*C. freundii*
27	Cucumber 2	*Ent. agglomerans*		

GENUS: *Ent.=Enterobacter; E.=Escherichia; Lecl.= Leclercia; Aer.=Aeromonas; K.=Klebsiella; C.=Citrobacter; Prov.=Providencia.*

L. monocytogenes was isolated from pre-packed mixed salads in UK (Sizmur & Walker, 1988) and from 7.8% of vegetables sold in markets in Barcelona, Spain (de Simón, Tarragó & Ferrer, 1992). In the current study, six aesculin-producing strains were isolated on Listeria selective agar from the vegetable samples. With API, the profiles were classified as doubtful or unacceptable to be classified as Listeria. These six strains were investigated further by employing the PCR technique (Chapter 4).

The Petrifilm method is very easy and convenient to use, but it does not allow easy access to specific colonies for confirmation, as may be required for the typing of enteropathogenic strains of *E. coli*. The time required to obtain results with petrifilm is similar to that of the conventional method (24 ± 2 h), but an additional 24 h may be necessary for slow-growing *E. coli* strains. Foschino, Caramaschi and Galli (1996), found that resuscitation (repair of damaged cells) of coliform bacteria in Tryptic soy broth for two hours before plating onto violet red bile agar (similar to MacConkey agar) significantly increased the recovery of coliforms. Standard count procedures do not incorporate any period for a cell recovery phase.

The use of conventional microbiological methods in the detection and identification of pathogenic bacteria is time-consuming and expensive. Not only the costs of the selective media and different enrichment stages but also the labour costs of the laboratory workers, attribute to these costs. In addition, pre-enrichment/enrichment broths, selective agars, reagents and confirmation kits have to be in stock. Most of the selective media must be prepared fresh and frequently (approximately every two to three weeks), and control strains must be constantly employed to batch test.

Although regulations state that food products for consumption should not contain any pathogens, regular laboratory testing for pathogens is not routinely performed throughout South Africa. The importance of pathogen detection is often overshadowed by the length of time a laboratory will take to correctly report a problem. Fresh milk or vegetable produce cannot be kept until pathogen results are available. Detection techniques for most pathogens take at least five days, and for *M. bovis* it takes at least six weeks.

CHAPTER 4

APPLICATION OF THE POLYMERASE CHAIN REACTION (PCR)

4.1 INTRODUCTION

The enrichment and selective media required for the resuscitation and isolation of diverse bacterial species that are considered potentially hazardous to health, isolated from milk and food sources such as vegetables, is extensive. There are also the additional requirements of identification systems and serological tests to characterise and confirm the presence of such bacteria. Procedures and times vary considerably, depending upon possible causative pathogens that have been implicated for a particular food source. For *Enterobacteriaceae*, perhaps two to three days is needed to fully confirm. Allowing the release and distribution of fresh milk produce and perishable foods for consumption before bacteriological results are known, is not an acceptable proposition. When one considers pathogens, often with delayed symptoms, that are capable of causing far more serious diseases, such as *Listeria* and *M. tuberculosis,* detection times of seven days to six weeks are totally unrealistic to be of any value to the food industry and public health.

New techniques have been introduced to enable shorter reporting periods. Many still take one to two days, require expensive equipment and/or reagent kits, highly technical expertise and are labour-intensive. Prime examples are enzyme-linked immunosorbent assays, hybridisation studies with gene probes and restriction endonuclease analysis (Beyi, Varfolomeeva & Tartakovskii, 1995; Blais *et al.,* 1997; Patel & Beuchat, 1995; Rodrigues *et al.,*1993; Shelef & Eden, 1996).

Of all the recent techniques for bacterial detection and genus/species identification, the polymerase chain reaction (PCR) holds most promise for rapid and sensitive confirmation of bacterial contamination of food products (Herman, de Blok & Moermans, 1995; Leal-Klevesas *et al.,* 1995). A further advantage of PCR is that, although specific primers are selected for individual bacterial species, all the other reagents are universal to the technique. The only other

variables are the temperature/time used for the actual PCR cycling conditions. The PCR has the added advantage over other recently applied methods in that it amplifies the specific target, enabling direct detection of even low numbers of bacterial cells.

A basic PCR technique involves: a) preparation of target DNA of good integrity without the presence of any PCR inhibitors; and b) amplification of the desired product which is achieved through PCR cycling. PCR cycling conditions vary, but comprise 1) denaturation of the template strands at 95°C; 2) primer annealing at suitable and specific temperatures, usually 45-65°C and 3) intervening DNA synthesis with a *Taq* polymerase at 72°C. The number of amplification cycles depends on the reaction mix composition, the length of product being produced and the detection limits required, normally between 25-40 cycles. The defined length of the PCR product is determined by the original sites at which each of the primers are directed. Depending on primer specificity, a single band on gel electrophoresis of the predicted size will be obtained (Hill, 1996).

The original selection of the target primer nucleotide sequences is the most critical aspect of PCR in that they should be unique to the genus or species that is being investigated. A variety of virulence determinants are known and custom-synthesised oligonucleotides have been produced that serve as primers of these genes. Since there are no international standards, primer choice and cycling conditions for a particular application are often difficult to make (Hill, 1996). It is important to take precautions to prevent any cross-contamination of previously amplified material to fresh samples that are about to undergo PCR, otherwise false positive results will be obtained (Hill, 1996). One general drawback of genetic techniques is that a positive result will be recorded even when the bacterium is non-viable. PCR is expensive, but the time factor is by far the most important advantage. Difficulties in implementing PCR for food analysis lies in problems encountered during template DNA preparation. Direct detection and concurrent specific identification, however, is achieved thereafter in one procedure. Theoretically a result would be forthcoming within a working day of six to seven hours. Important candidates for PCR detection are *Brucella*, *Campylobacter*, *Listeria* and mycobacteria.

4.2 RESULTS AND DISCUSSION

4.2.1 PCR Identification

PCR detection limits employing *L. monocytogenes* specific primers (LM1/2) with two dynabead lysis protocols were determined on cell suspensions of known cell numbers in milk (Fig. 4.1). Cell lysis with heating at 65°C for 15 min enabled detection equivalent to one cell and lysis for 15 min at 25°C 10 cells per 1.5 µl of target DNA addition. For all further dynabead DNA extractions heating for 15-30 min at 65°C was performed.

From the results obtained in Chapter 3, using conventional methodology, *L. monocytogenes* was isolated from four milk samples: 16 cow, 18 raw batch from the same farm and day as sample 16 was taken, 36 depot pasteurised and 44 a raw batch (Table 3.2). PCR was applied to these API identified *L. monocytogenes* strains with specie specific LM1/2 primers in conjunction with universal primers U1/2. The universal primers U1/2 were tested for their future potential use in studies on milk as a control to ensure samples and PCR conditions are correct. *L. monocytogenes* PCR products of approximately 702 bp were demonstrated from strains 16 (very weak), 18, 36 and 44 (Fig 4.2). No PCR product of approximately 408 bp was seen with the universal primers for strains 16 and 18 in a second experiment indicating target DNA was not satisfactory or cycling was faulty for those tubes.

The six presumptive *Listeria* strains isolated from vegetables (Chapter 3), with the exception of one, all had unacceptable/doubtful *Listeria* genus API profiles (Table 4.1). On applying *L. monocytogenes* specific and universal primers to these strains, insufficient lysis was noted for three strains (11, 18, 23). However, a PCR product of approximately 702 bp indicative of *L. monocytogenes* was demonstrated from strain 33 isolated from beans, and strain 21 isolated from lettuce (Fig. 4.3).

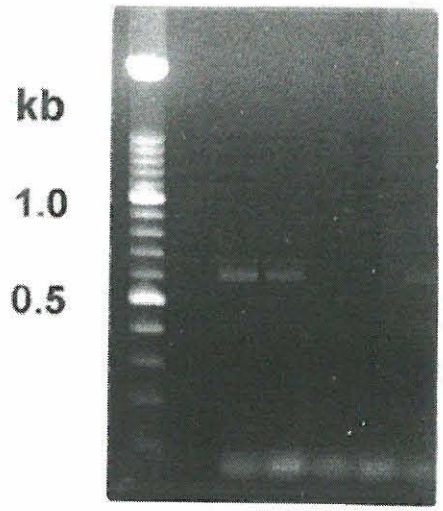

Fig. 4.1 Detection limits of *L. monocytogenes* based on theoretical cell numbers (TCN) calculated from an initial cell suspension of 1 X 10^7cfu/ml. Lysis method A: dynabead lysis mix 25°C, 15 min; method B:dynabead lysis mix 65°C, 15 min.
LANES: 1, MWM XIV; 2, water negative control; 3, lysis method A TCN 1; 4, lysis method A TCN 10; 5, lysis method B TCN 0.1; 6, lysis method B TCN 1; 7, lysis method B TCN 10.

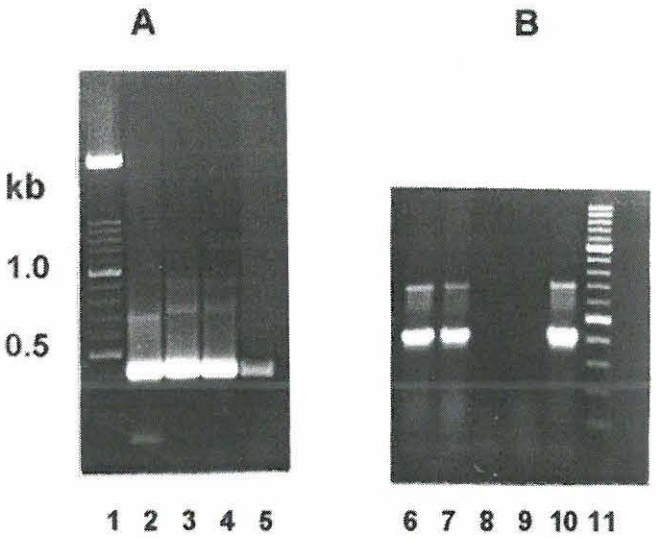

Fig. 4.2 Confirmation of *L. monocytogenes* cultures and PCR reproducibility employing universal (U1/2) and specific *L. monocytogenes* (LM1/2) primers. A and B were independent experiments.
LANES: 1, MWM XIV; 2, *L. monocytogenes* control culture; 3, isolated from milk 44; 4, isolated from milk 18; 5, isolated from milk 16; 6, isolated from milk 44; 7, isolated from milk 36; 8, isolated from milk 18; 9, isolated from milk 16; 10, *L. monocytogenes* control culture; 11, MWM XIV.

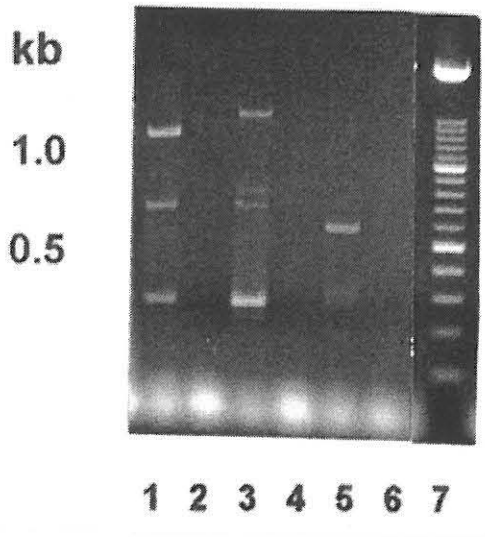

Fig. 4.3 Confirmation of *L. monocytogenes* cultures employing universal (U1/2) and specific *L. monocytogenes* (LM1/2) primers.
LANES: 1, isolated from beans sample 33; 2, isolated from spinach sample 23; 3, isolated from lettuce sample 21; 4, isolated from beans sample 11; 5, isolated from spinach sample 10; 6, isolated from mushrooms sample 8; 7, MWM XIV.

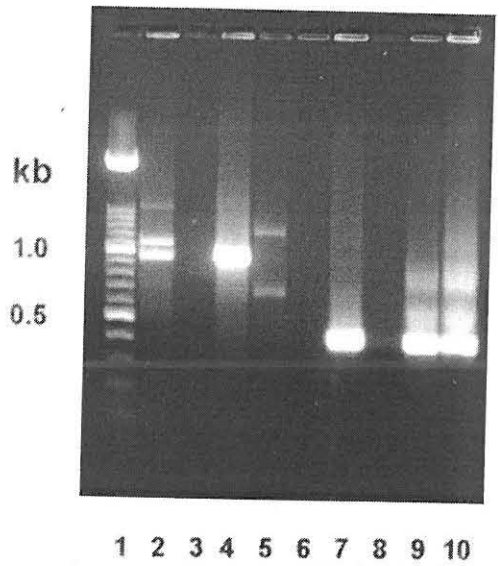

Fig. 4.4 Confirmation of *Campylobacter jejuni* cultures employing two primer sets CJ3/4 lanes 2-5 and CJ1/2 lanes 6-10.
LANES: 1, MWM XIV; 2, isolated from milk 38, CJ3/4; 3, isolated from milk 10, CJ3/4; 4, isolated from milk 6, CJ3/4; 5, isolated from milk 4, CJ3/4; 6, *C. jejuni* "control" culture, CJ1/2; 7, isolated from milk 38, CJ1/2; 8, isolated from milk 10, CJ1/2; 9, isolated from milk 6, CJ1/2; 10, isolated from milk 4, CJ1/2.

Table 4.1 API profiles of presumptive *L. monocytogenes* strains isolated from vegetable samples.

Vegetable (No.)	Profile/Description	Strain
Mushroom (8)	2710 acceptable to genus	L. ivanovii 59% / L. welshimeri 28%
Spinach (10)	6600 unacceptable	L. welshimeri ,/ L. monocytogenes / L. innocua
Beans (11)	7610 doubtful	L. welshimeri 65.6% / L. innocua 34%
Lettuce (21)	7610 doubtful	L. welshimeri 65.6% / L. innocua 34%
Spinach (23)	7600 unacceptable	L.welshimeri ./ L. innocua
Beans (33)	6610 unacceptable	Genus Listeria not given

Often results from conventional tests or API substrates are dependent on 65%-99% of strains being positive. Conversely, it is not known if PCR products of a predicted size are all 100% specific. It is necessary to test local strains and continuously correlate and update PCR performance.

Isolates from campylobacter medium that were not confirmed as *Campylobacter* species, using the API system, were also included for PCR identification studies, using two primer pairs CJ1/2 and CJ3/4 (Fig. 4.4). Only non-specific bands were seen for all strains, except for a weak product of predicted size, 710 bp, being evident with CJ3/4 primers for milk 38 but no band of 256 bp was demonstrated with the primers CJ1/2 (Fig. 4.4 lanes 2 and 7). A reported "control" culture produced no bands and, although an extensive search through *C. jejuni* stock cultures at the Microbiology Department, Universitas Hospital, was made, they proved to be non-viable.

PCR on a lysed control *B. abortus* strain employing primer pairs B1/2 and B3/4, produced the species specific bands of approximately 489 bp and 905 bp respectively.

4.2.2 PCR Detection

Having confirmed as *L. monocytogenes* the four isolates from milk, direct and dynabead PCR detection was applied to these original four milk samples (Fig. 4.5). A PCR product of approximately 600 bp was obtained, using the direct system from milk samples 16 and 36 (both very weak bands) and 44. When using the dynabead concentration kit, a very strong band was found for milk 16. It was immediately evident that a) the control lysate did not produce a *L. monocytogenes* specific band due possibly to lysate degradation; b) no universal product was seen for milk 44; and c) the PCR products were approximately 600 bp, and not 700 bp as expected.

It was therefore necessary to investigate the effect of a) milk as a medium; b) dynabeads as a concentration step; and c) combined *L. monocytogenes* and universal primers on PCR products generated.

The effect of different unpasteurised and pasteurised milks and cell viability was investigated by inoculating milk with *L. monocytogenes* at a final concentration of 2-5x10^4 cfu/ml. The inoculated milk was then pasteurised at 63°C for 30 min (P1) and repasteurised at 63°C for 90 min (P2). Samples were then taken from the milk immediately and at time periods of 24 h, 48 h, 72 h and 7 days, after holding the milks at 25°C and 4°C (Figs. 4.6 and 4.7). The PCR results are summarised in Table 4.2. Interestingly, the initial cell count of 2-5x10^4 cfu/ml *L. monocytogenes* resulted in incomplete pasteurisation, as viability was still recorded at 80 cfu/ml. For repasteurised milk at 0 h and after 7 days at 4°C, *L. monocytogenes* viability was <10 cfu/ml, although target DNA remained intact as PCR products of 600-700 bp were still demonstrated (Fig 4.6 lanes 5, 6 and Fig 4.7 lane 29).

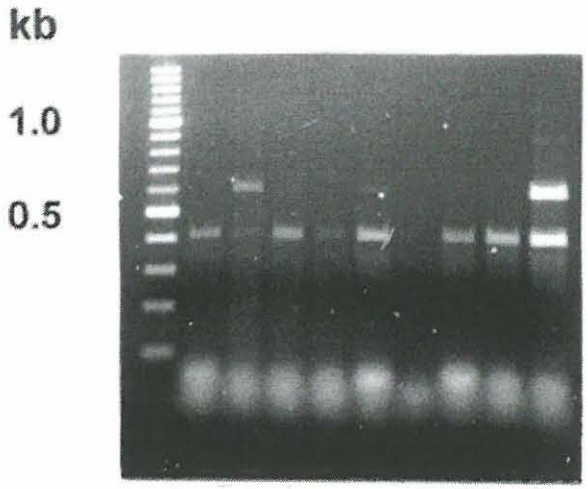

Fig. 4.5 Detection of *L. monocytogenes* directly from milk and by employing the Dynabead DNA concentration kit.
LANES: 1, MWM XIV; 2, *L. monocytogenes* control culture; 3, milk sample 44 direct; 4, milk sample 36 direct; 5, milk sample 18 direct; 6, milk sample 16 direct; 7, Dynabead milk sample 44; 8, Dynabead milk sample 36; 9, Dynabead milk sample 18; 10, Dynabead milk sample 16.

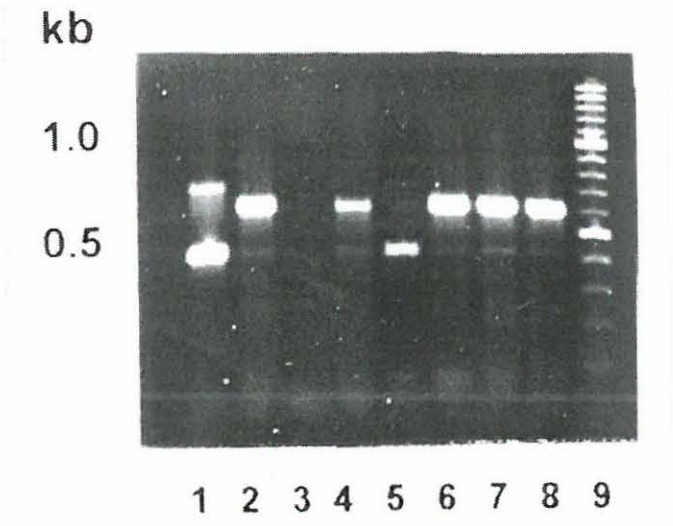

Fig. 4.6 Technique standardisation employing universal/ *L. monocytogenes* primers and the Dynabead concentration kit. Milk was inoculated with *L. monocytogenes* to 2-5x10^{4}cfu/ml. A sample of the inoculated milk was pasteurised at 63°C for 30 minutes (P1) and repasteurised at 63°C for 90 minutes (P2). Both unpasteurised and pasteurised milk was held at 25°C and 4°C for 0-24 hours
LANES: 1, unpasteurised-0h; 2, P1-0h; 3, P1-24h 4°C; 4, P1-24h 25°C; 5, P2-0h; 6, P2-0h, direct; 7, P2-24h 4°C; 8, P2-24h 25°C; 9, MWM XIV.

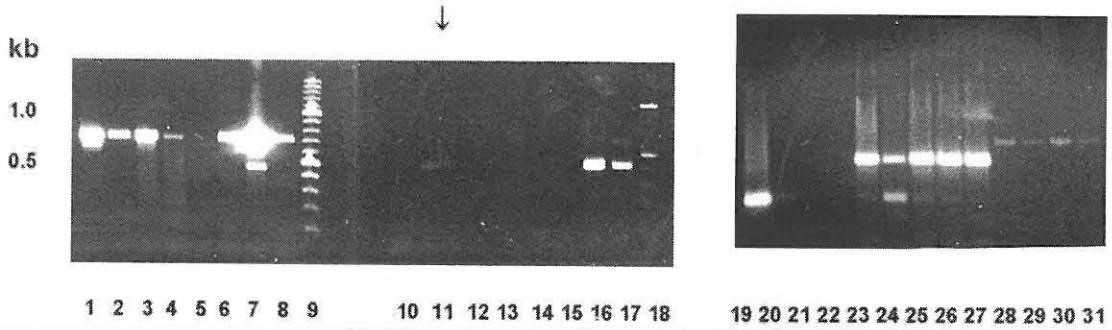

Fig. 4.7 Dynabead technique standardisation using universal and *L. monocytogenes* primers independently and combined. Universal (U1/2), lanes 11 to 17, *L. monocytogenes* (LM1/2) primers, lanes 1 to 8 and 28 to 31 and lanes 19 to 27 in combination Abbreviations concerning milk status unpasteurised and pasteurised are described in the legend of Figure 4.6.
LANES: 1, P2-72h 25°C; 2, P2-72h 4°C; 3, P1-7days 25°C; 4, P1-7days 4°C; 5, inoculated milk-7days 25°C; 6, inoculated milk-7days 4°C; 7, inoculated milk-48h 25°C; 8, inoculated milk-48h 4°C; 9, MWM XIV; 10, P2-72h 25°C; 11, P2-72h 4°C; 12, P1-7days 25°C; 13, P1-7days 4°C; 14, inoculated milk-7days 25°C; 15, inoculated milk-7days 4°C; 16, inoculated milk-48h 25°C; 17, inoculated milk-48h 4°C; 18, MWM XIV; 19, milk 43; 20, milk 29; 21, milk 43; 22, milk 29; 23, P1-72h 4°C; 24, P2-7days 4°C; 25 P2-72h 25°C; 26, P1-0h; 27, inoculated milk-0h; 28, P1-7days 4°C; 29, P2-7days 4°C; 30, P2-72h 25°C; 31, P1-0h.

PCR products from the universal primers were inexplicably not present for some milk samples (Fig. 4.6 lane 3, Fig. 4.7 lanes 12-15, 18, 21, 22). Apart from the 0 h inoculated sample (Fig. 4.6 lane 1), whenever U1/2 primers were combined with LM1/2 primers, *L. monocytogenes* specific PCR products of 600 bp were observed and not 700 bp as expected. At this time the size difference did not appear to be dynabead-related, as can be seen by comparing Figure 4.6 lane 5, dynabead with Figure 4.6 lane 6, direct. In both cases a product of 600 bp was recorded. An indication as to storage problems was beginning to emerge in that the 0 h milk sample produced a 700 bp PCR product (Fig. 4.6 lane 1). However, after storage with dynabeads at –20°C and re-thawing on re-amplification this same milk sample gave rise to a 600 bp product (Fig. 4.7 lane 27).

Table 4.2 **Summary of PCR products obtained after different heat treatments and storage conditions employing *L. monocytogenes* primers independently and in combination with universal primers** (Figs. 4.6 and 4.7). Dynabeads were used for all milk samples except for P2 direct Fig.4.6 lane 7.

Milk treatment	Storage	Fig.	Lane	PCR products		
				U1/2 (400 bp)	LM1/2 (600 bp)	LM1/2 (700 bp)
I		4.6.	1	+		+
P1		4.6.	2	+	+	
P1	25°C, 24 h	4.6.	4	+	+	
P2		4.6.	5	+	+	
P2 direct		4.6.	6	+	+	
P2	4°C, 24 h	4.6.	7	+	+	
P2	25°C, 24 h	4.6.	8	+	+	
P2	25°C, 72 h	4.7.	1	U-		+
P2	4°C, 72 h	4.7.	2	U-		+
P1	4°C, 7days	4.7.	3	U-		+
P1	4°C, 7days	4.7.	4	U-		+
I	25°C, 7days	4.7.	6	U-		+
I	25°C, 48 h	4.7.	7	U-		+
I	4°C, 48 h	4.7.	8	U-		+
P2	4°C, 72 h	4.7.	11	+		
I	25°C, 48 h	4.7.	16	+		
I	4°C, 48 h	4.7.	17	+		
Milk 43		4.7.	19	+		
Milk 29		4.7.	20	+		
P1	4°C, 72 h	4.7.	23	+	+	
P2	4°C, 72 h	4.7.	24	+	+	
P2	25°C, 72 h	4.7.	25	+	+	
P1		4.7.	26	+	+	
I		4.7.	27	+	+	
P1	4°C, 7days	4.7.	28	U-		+
P2	4°C, 7days	4.7.	29	U-		+
P2	25°C, 72 h	4.7.	30	U-		+
P1		4.7.	31	U-		+

U- = No universal primers U1/2 used.

I = Inoculated with *L. monocytogenes* to 2.5×10^4 cfu/ml, no heat treatment.

P1 = Milk inoculated and pasteurised 63°C for 30 min.

P2 = Milk inoculated and repasteurised at 63°C for 90 min.

Experimentation was continued to investigate possible cross-primer product formation by employing primer LM1/U2 and U1/LM2 combinations. No PCR products were observed with these primer formats on lysed *L. monocytogenes* cells (Table 4.3).

Table 4.3 Investigation into primer combinations on PCR product size

Milk treatment		Primer combination			
		U1/LM2	LM1/U2	LM1/2	U1/2
Unpasteurised	day 1	-	-	-	+
Pasteurised	day1	-	-	+(700 bp)	+
Repasteurised	day 7	-	-	+(700 bp)	+
Repasteurised	day 11	-	-	+(700 bp)	-
Pasteurised	4°C, day7	-	-	+(700 bp)	-
Milk 29				+(600 bp)	+
Milk 43				+(600bp)	+

At this stage a group of milk samples were screened for the presence of *L. monocytogenes* specific DNA (Fig. 4.8, Table 4.4). It is clearly evident that there was PCR failure with some milk samples, as a universal PCR product was not demonstrated. A PCR product of 600 bp was seen with four milks as a result of possible *L. monocytogenes* cells/DNA. Additional milk samples were screened with universal primers, but few PCR products were obtained.

Table 4.4 Screening for the presence of *L. monocytogenes* in milk samples

Milk	Sample No.	PCR products	
		U1/2 primers (400 bp)	LM1/2 primers (600 bp)
Cow	19	+	+
Cow	20	+	+
Cow	21	-	-
Raw batch	29	-	-
Raw batch	30	-	-
Raw batch	32	+	+
Depot	34	-	-
Depot	35	+	+
Depot	37	+	-
Raw batch	39	-	-
Depot	40	+	-
Raw batch	41	+	-
Raw batch	43	-	-

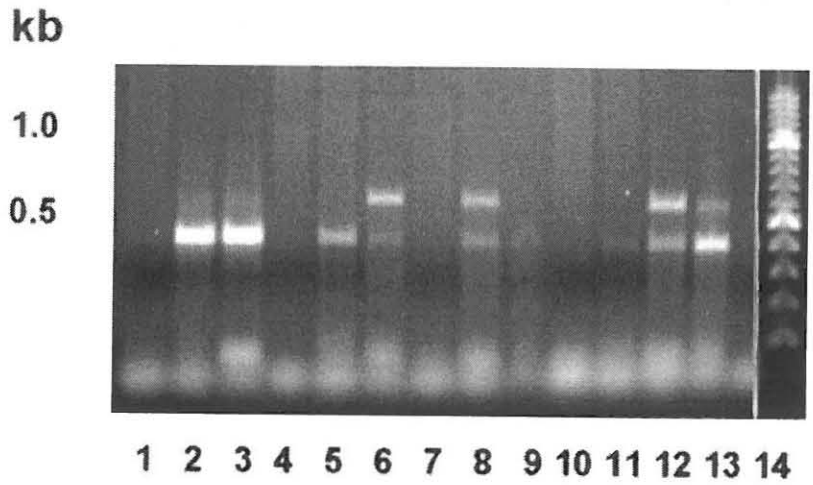

Fig. 4.8 Detection of *L. monocytogenes* in milk samples employing the Dynabead concentration kit.
LANES: 1, milk 43; 2, milk 41; 3, milk 40; 4, milk 39; 5, milk 37; 6, milk 35; 7, milk 34; 8, milk 32; 9, milk 30; 10, milk 29; 11, milk 21; 12, milk 20; 13, milk 19; 14, MWM XIV.

Further investigations as to why milk samples were not seen to be amplifying, were conducted. Milk samples that had basically "failed" with the universal primers, were tested to determine whether DNA degrading enzymes were present. The milks were spiked with 3 µl of MWM XIV and incubated at 25°C for 1 h (Fig. 4.9). As difficulties were also being experienced with dynabead re-amplification, dynabead/DNA/residual milk stored suspensions were similarly spiked with MWM XIV (Fig. 4.9). The DNA remained intact in all cases but it could be seen that bands of the same size were migrating at slightly different rates. This apparent shift to lower molecular size was evident with the dynabead preparations (Figure 4.9, lanes 6, 7and 9 and UHT milk lane 1). The only explanation is multi-complexing of the DNA with milk components and/or the dynabeads themselves.

The pH of samples that had not performed, was determined in case binding of DNA to the dynabeads was reduced under acidic conditions as a result of a high bacterial content. The difference in pH was negligible, ranging from pH 6-7 for milk samples that did or did not perform.

The final concept considered within the time frame of this study for non-reproducible results was that of freeze/thaw. New milk samples with high bacterial counts were taken and the influence of freezing and thawing and the number of times this aspect occurred, were investigated (Fig. 4.10, Table 4.5). Again dynabead preparations were also subjected to similar analysis (Fig. 4.10, Table 4.5). Freezing and thawing of the milk samples 1 and 3 and freezing/thawing of milk 1 dynabeaded preparations, seriously affected PCR results. This was unexpected, and it is recommended that retrospective studies should not be conducted unless the DNA preparation is totally free of milk components and can be treated as standard purified DNA.

The introduction of PCR techniques in testing milk products still remains a viable option for immediate analysis with the understandable, yet complicated problem, that predicted product size may not necessarily be realised.

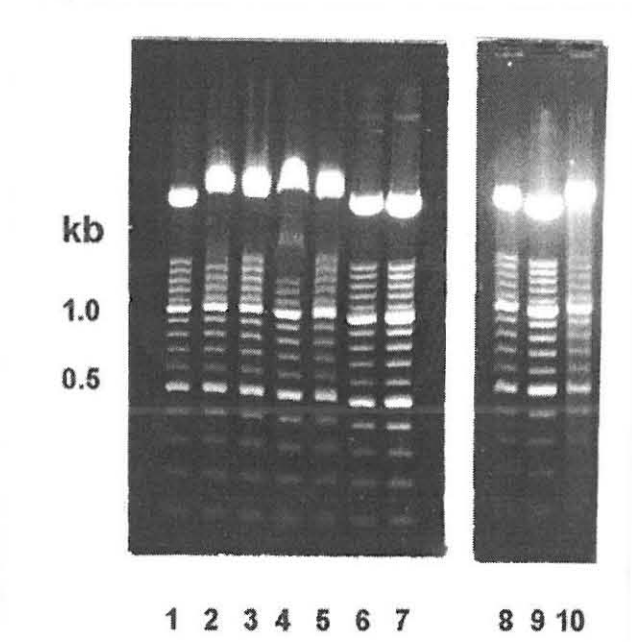

Fig. 4.9 Technique standardisation based on the spiked addition of molecular weight marker XIV to different milk samples.
LANES: 1, UHT milk; 2, milk 47; 3, milk 46; 4, milk 19; 5, milk 10; 6, milk 46, Dynabead; 7, milk 19, Dynabead; 8, UHT milk; 9, milk 46 Dynabead; 10, milk 46.

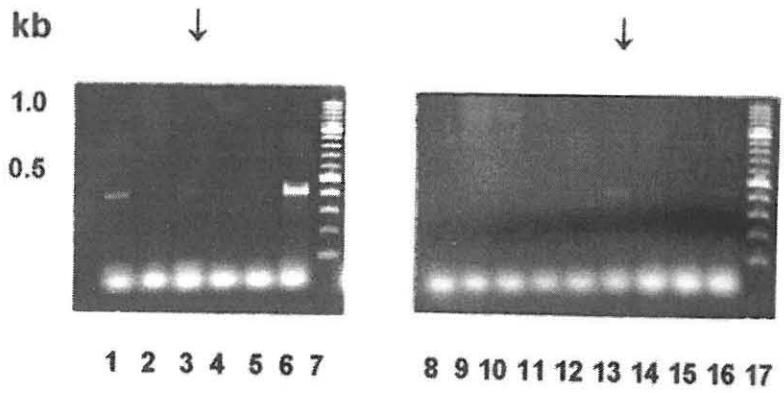

Fig. 4.10 Effect of freezing and thawing on PCR detection of *L. monocytogenes*.
LANES: 1, full cream milk inoculated with *L. monocytogenes,* heated at 99°C for 10 minutes; 2, low fat milk sample, heated at 99°C for 10 minutes; 3, full cream milk sample, heated at 99°C for 10 minutes; 4, milk inoculated with *L. monocytogenes, Dynabead*; 5, low fat milk sample, Dynabead; 6, full cream milk sample, Dynabead; 7, MWM XIV; 8, milk inoculated with *L. monocytogenes,* froze/thawed x2, heated at 99°C for 10 minutes; 9, low fat milk sample, froze/thawed x2, heated at 99°C for 10 minutes; 10, full cream milk sample, froze/thawed x4, heated at 99°C for 10 minutes; 11, milk inoculated with *L. monocytogenes,* Dynabead sample froze/thawed once; 12, low fat milk sample, Dynabead sample froze/thawed once;13, full cream milk sample, Dynabead sample froze/thawed once; 14, milk inoculated with *L. monocytogenes,* froze/thawed once, Dynabead; 15, low fat milk froze/thawed once, Dynabead; 16, full cream milk sample froze/thawed x3, Dynabead; 17, MWM XIV.

Table 4.5 Effect of freezing/thawing on the demonstration of PCR products from milk and dynabead preparations using the universal primers U1/2 (Fig.4.10)

Milk	Treatment	Detection	PCR product
Milk 1		Dynabead	+
Milk 2		Dynabead	-
Milk 3		Dynabead	-
Milk 1		Direct	+
Milk 2		Direct	-
Milk 3		Direct	+
Milk 1	3x freeze/thaw	Dynabead	-
Milk 2	1x freeze/thaw	Dynabead	-
Milk 3	1x freeze/thaw	Dynabeads	-
Milk 1	4x freeze/thaw	Direct	-
Milk 2	2x freeze/thaw	Direct	-
Milk 3	2x freeze/thaw	Direct	-
Milk 1 Dynabead	1x freeze/thaw	Re-eluted	+ very weak
Milk 2 Dynabead	1x freeze/thaw	Re-eluted	-
Milk 3 Dynabead	1x freeze/thaw	Re-eluted	-

4.3 CONCLUSIONS

The conventional microbiological and PCR results concerning the presence of *L. monocytogenes* in four milk samples investigated were in accordance. Possible *L. monocytogenes* in four other milk samples, was also indicated by the PCR method. Two vegetables, from which presumptive *Listeria* species were isolated, did not correlate with PCR identification. A PCR product confirming *L. monocytogenes* (primers directed against the specific listeriolysin o gene *of L. monocytogenes*) was found, but the API profiles failed to produce a definite classification. The reverse can also be applied. It is necessary to test primers

that have been designed and tested in the USA or Europe as specific to their strains, against local isolates because primer mismatch may occur. In addition, there is always the possibility that a non-specific product of approximately the same size as that expected may be resolved on electrophoresis with the misinterpretation of a positive PCR result. The two primer pairs selected for detecting *B. abortus* appeared excellent, but due to problems encountered during the study, their full value could unfortunately not be evaluated.

PCR from the onset and with the enormity of scientific attention received, appears a very attractive method for the detection of foodborne pathogens. It was found, however, that milk was not an easy medium to incorporate into the PCR system as a) there was tremendous difficulty in obtaining good reproducible target DNA, as universal primers were employed; and b) PCR product sizes were not always as expected. The universal and *L. monocytogenes* primers/combinations and conditions were exactly as those described and recommended by Siggens (1995). The dynabead DNA concentration concept which is vital for the detection of bacteria at low cell numbers and to exclude inhibitory substances from samples, is brilliant, but performance of the beads was certainly influenced by unknown components present in milk and they introduced yet another variable into the system.

Even under the strictest of controlled conditions and in the most competent hands, PCR can go inexplicably astray. Once a problem becomes evident, it is extremely difficult to locate the source and it is very time-consuming to exclude. Each component of the PCR reaction has to be methodically and sequentially checked for a) DNase contamination of any one or more PCR reaction mixture constituents; b) possible variations/inhibitory aspects of individual samples; c) *taq* batch variability; d) primer synthesis purity; and e) malfunction of the PCR machine. Although PCR technology can be fraught with inherent problems, it remains the most comprehensive and convenient option for future rapid detection/ identification of foodborne pathogens.

CHAPTER 5

ANTIBIOTIC RESISTANCE

5.1 INTRODUCTION

Resistance to antimicrobial agents can be accomplished in two general ways, namely by a) mutation(s) in the existing DNA of the bacterium or b) the acquisition of new DNA carrying a resistance trait (Murray & Holden-Christian, 1991).

Spontaneous mutations are an ongoing occurrence within bacterial DNA, with specific mutations proving a selective advantage over other bacteria, as is the case for antibiotic resistance development. Examples of resistance development as a result of mutations that have been shown to have important clinical implications are a) ribosomal alterations giving rise to aminoglycoside resistance; b) penicillin-binding protein (PBP) alterations that reduce binding affinity of ß-lactam antibiotics; c) alterations to DNA gyrase reducing interaction of quinolones; d) alterations of outer membrane permeability reducing quinolone and ß-lactam uptake; and e) mutations in acquired genes, including those of plasmids and transposons, that can create new resistance phenotypes (Murray & Holden-Christian, 1991). This is especially evident in the emergence of extended spectrum ß-lactamases which are capable of hydrolysing third-generation cephalosporins.

The means by which resistance genes can be acquired, are a) transformation: transfer and integration of DNA for example resistance gene blocks of PBPs to species that are naturally transformable; b) transduction: transfer of DNA by phages for example staphylococcal ß-lactamase; c) conjugation: transfer of plasmid containing antibiotic resistance genes between species; and different genera depending on the plasmid host range and d) transposition: movement of DNA by transposition and recombination, transposons having a broad host range

(Berkowitz, 1995). Many resistance determinants may be interchangeably plasmid or chromosomal, because they are carried on transposable elements. Resistance genes that are present on plasmids/transposons, are numerous and usually encode enzymes that are able to inactivate a number of antibiotics within an antimicrobial group, such as ß-lactamases and aminoglycoside modifying enzymes. This type of resistance is usually high-level as the enzyme hydrolyses or modifies an antibiotic, rendering it totally ineffective. Resistance determinants can also influence antibiotic accumulation in the cell by means of promoting active efflux, as found with tetracycline (Murray & Holden-Christian, 1991).

In the environmental setting, a constant low-level exposure to antimicrobial agents can lead to the development of resistance due to mutations. The use or abuse of antibiotics with constantly imposed selective pressures can result in multi-drug resistance acquisition by the accumulation of plasmids/transposons which can then in turn be disseminated to other bacteria.

Problems are evident in administering antibiotics for effective mastitis treatment. Interactions between the host and pathogens have been recognised and they include a) poor penetration of antibiotic into mammary tissues; b) intracellular location of bacteria in leucocytes of mammary tissues or micro-abscesses; c) reduced antibiotic activity in milk; and d) the development of resistance by the bacteria as therapy proceeds (Owens, Ray & Washburn, 1993; Louhi-Lethiö *et al.,* 1994). The above situations that are known to affect effective treatment, could encourage farmers and veterinary surgeons to employ perhaps more effective antibiotics with perhaps less protein binding. It would be the most recent released or approved agents for multi-drug-resistant bacteria causing human infections. In addition, veterinary papers advocating the use and success rate of antibiotics that should be reserved for hospitals, abound. Quinolones were first introduced in 1983, after which resistance developed rapidly in some bacteria. Ciprofloxacin still remains very effective, yet it is promoted for the treatment of mastitis caused by gram-

negative bacteria. A study by Shpigel *et al.* (1997), in Israel supported the efficacy of cefquinome with reduced milk loss for treating *E. coli* mastitis. Cefquinome is, however, an advanced broad-spectrum cephalosporin that resists β-lactamase activity by the majority of clinically important bacteria. Antimicrobial agents are also administered *ad hoc* prophylactically in order to keep herds in a good condition. Such agents if (or even should be) used, must not have clinical application (Wray, 1997). Avoparcin was employed as a growth promoter in Europe and the USA, but its structural resemblance to vancomycin, which is a last-line antibiotic for methicillin-resistant staphylococci, caused its withdrawal due to linked cross-resistance being observed (Wray, 1997). Another agent in use is apramycin, which can give rise to cross-aminoglycoside resistance. There is evidence that resistant bacterial strains can be spread from animals to humans as an apramycin-resistant *E. coli* was isolated from handlers working with pigs, after an outbreak in a pig unit (Johnson, 1997).

It has become more and more evident that previously susceptible organisms have become resistant (Berkowitz, 1995). It can be expected that the type and degree of resistance development or maintenance in a specific area will be a product of the antimicrobial agents in common usage, whether it be hospital, general practitioner, veterinarian or farmer. Only a limited number of studies have been conducted to investigate antibiotic resistance gene pools present in bacteria isolated from milk and environmental sources (Personal communication – LJ Chalkley). Antibiotic resistance of bacteria from farm locations and the Bloemfontein environment is unknown.

5.2 RESULTS AND DISCUSSION

All milk, vegetable and farm environmental samples were investigated for bacteria that may have developed or acquired antibiotic resistance genes. The antibiotics were selected on the basis of their clinical use and divided into two series as to their known efficacy against gram-positive and gram-negative bacteria.

As a pilot study, milks were initially screened on antibiotic selective media to estimate the general extent of antibiotic resistance before proceeding to perform further in-depth studies. It can be seen from Table 5.1 that resistance was extensive, even exceptionally high in the non-lactose fermenting gram-negative bacteria.

Table 5.1 Screening of milk samples to assess degree of resistance in gram negative bacteria

Sample	Total count (cfu/ml)	Bacterial type	Growth on antibiotic containing media (cfu/ml)							
			AMP 16	CHL 16	CFOX 16	CTAX 16	GEN 8	KAN 16	STR 8	TET 8
27	83	LF	30	0	0	0	0	3	52	75
	43	NLF	4	2	3	4	0	10	3	2
29	140	LF	20	0	4	0	0	0	0	6
	300	NLF	300	250	300	300	9	21	250	32
30	50	LF	5	0	3	0	0	0	1	0
	120	NLF	120	0	90	83	45	80	120	105
31	40	LF	11	0	6	0	0	0	0	0
	139	NLF	60	18	95	22	1	20	45	4
32	43	LF	37	0	21	0	0	0	5	0
	350	NLF	200	0	260	16	2	140	200	200

LF=Lactose fermenter. NLF=Non-lactose fermenter

Enterococci and staphylococci were isolated from plates containing 8 µg/ml vancomycin, but on performing MIC determinations their MICs were ≤ 0.5 µg/ml. This emphasises problems encountered with resistance screening as, in effect, the initial isolate was exhibiting the tolerance phenomenon and not the presence of a vancomycin-resistance gene.
Because of the many problems associated with selective screening, individual strains were haphazardly sampled and subjected to controlled resistance breakpoint determinations.

The resistance profiles of staphylococci isolated from raw and pasteurised milk during 1997 and 1998 are shown in Table 5.2. Of 14 *S. aureus* strains isolated in 1997, 7 strains were resistant to three or more antibitotics with streptomycin, tetracycline and cephalosporin resistance evident. In 1998 cefoxitin resistance was still found as was ciprofloxacin- and oxacillin-resistant isolates. The majority of antibiotic-resistant CNS carried resistance to ciprofloxacin, streptomycin and, disturbingly, oxacillin (Table 5.2).
A study conducted in Zimbabwe in 1996 on milks obtained from large-scale commercial dairies, small-scale commercial dairies and communal dairies, found that 69% of *S. aureus* isolates (100 strains) and 36 strains (66%) of CNS were resistant to one or more antibiotics. The most common resistance combinations were streptomycin/oxytetracycline and penicillin/oxytetracycline (Makaya, Aarestrup & Olsen, 1996). In the current study conducted during 1997, streptomycin and tetracycline resistance were also seen in conjunction. *S. aureus* strains isolated from mastitic milk in Trinidad, revealed that 85 of 250 strains (34%) were resistant to antimicrobial agents, of which 59 were resistant to penicillin and 44 strains showed resistance to ampicillin, but only three to methicillin (Adesiyun, 1995). Penicillin resistance was not as prevalent as one might have thought in the staphylococci investigated, perhaps indicating that South Africa has moved towards more recent antibiotics, as reflected in cephalosporin and ciprofloxacin resistance levels (Table 5.2).

Table 5.2 Resistance profiles of staphylococci isolated from milk

Sample	Species	No. of strains	Antibiotic resistance profiles
1997			
Cow	*S.aureus*	3	Fully susceptible
	S.aureus	1	ERY, STR, TET,
	S.aureus	1	CFOX, CTRX, GEN, KAN, RIF, STR,TET,
	S.aureus	2	CFOX, CTAX, CTRX, ERY, KAN, STR, TET
	CNS	1	CFOX, ERY, KAN, RIF, STR
	CNS	1	AMP, CFOX, ERY, PEN, RIF
Raw batch	*S.aureus*	3	Fully susceptible
	S.aureus	2	PEN
	S.aureus	1	CFOX, CTAX, CTRX, ERY, KAN, STR
	CNS	5	Fully susceptible
	CNS	3	STR
	CNS	1	TET
	CNS	1	PEN, STR
	CNS	1	STR, TET
	CNS	1	CIPX^{I}, OXAC
	CNS	1	CIPX^{I}, OXAC, STR
	CNS	2	CIPX, OXAC, STR, TET
	CNS	2	KAN, CIPX, OXAC, STR
	CNS	2	CIPX^{I}, ERY, GEN, OXAC, PEN, RIF, SMX/TMP, STR, TET
Past batch	*S aureus*	1	CFOX, GEN, KAN, STR
	S.aureus	1	CFOX, CTAX, CTRX, GEN, KAN, STR, TET
	CNS	1	Fully susceptible
	CNS	3	CIPX, OXAC, STR
	CNS	4	CIPX, OXAC, STR, KAN
	CNS	2	CIPX, OXAC, STR, SMX/TMP^{I}
	CNS	1	CIPX, OXAC, STR, TET
	CNS	2	CIPX, GEN, KAN, OXAC, STR, SMX/TMP^{I}
	CNS	1	CIPX, KAN, OXAC, STR, SMX/TMP^{I}, TET

Table 5.2 continued

1998			
Raw batch	***S.aureus***	**5**	**Fully susceptible**
	S. aureus	**1**	**CFOX, ERY, PEN, RIF**
	CNS	**7**	**Fully susceptible**
	CNS	**1**	**CFOX, CIPX^{I}, OXAC**
	CNS	**1**	**CFOX, ERY, PEN, OXAC, RIF**
Past batch	***S. aureus***	**1**	**CFOX, CIPX^{I}, GEN, OXAC**
	S. aureus	**1**	**CFOX, CIPX^{I}, GEN, OXAC, TET**
	CNS	**4**	**Fully susceptible**
	CNS	**1**	**CFOX, GEN, OXAC**
	CNS	**1**	**CFOX, CIPX^{I}, GEN, OXAC**
	CNS	**1**	**CFOX, ERY, OXAC, PEN**

CNS = coagulase negative staphylococci Past=Pasteurised

The antibiotic resistance profiles of gram-negative bacteria isolated from milk samples are shown in Table 5.3, and the number of strain types that exhibited resistance to three or more antibiotics are summarised in Table 5.4. Results show that multiplicity of resistance is overwhelming, and certainly veterinarians would probably have major problems trying to successfully treat gram-negative bacterial mastitis. Ampicillin and augmentin, which is still used by general practitioners for human infections, are likely to be ineffective. Of the NLF strains isolated in 1997, 9/14 strains were resistant to eight or more antibiotics and all three *Stenotrophomonas maltophilia* strains were resistant to nine or more antibiotics.

E. coli strains were isolated from raw milk in Nairobi, Kenya, and 95% of the isolates were resistant to sulphamethoxazole with 29% of these strains showing multiple resistance. The frequency of resistance to tetracycline was 12%, cotrimoxazole 10% and streptomycin 10% respectively (Ombui, Macharia & Ndhuhiu, 1995). This revelation is quite mild in comparison to the findings of this study.

Obviously any resistant bacteria that are introduced into milk have the possibility of surviving the pasteurisation process, especially if regulated raw milk counts are exceeded. It is not known if isolation and susceptibility testing is performed on suspected bacteria from mastitis cases. If an empiric approach is generally used, it is possible that the most recent antibiotics would be employed against gram-negative bacteria.

The farm environmental isolates and the strains tested from vegetables did not appear to be as multiple resistant to those from milk produce (Tables 5.6 and 5.5), the major exceptions being *Enterobacter* and *Pseudomonas* species, although, as found for the milk samples, ampicillin and augmentin resistance were high.

Table 5.3. Resistance profiles of gram negative bacteria isolated from milk

Sample	Bacterial type	No. of strains	Antibiotic resistance profiles
1997			
Cow	LF	4	Fully susceptible
	LF	1	TET
	LF	1	AMP, CFOX
	LF	1	AMP, CFOX, CHL
Raw	LF	4	Fully susceptible
batch	LF	2	AMP
	LF		CHL
	LF	2	CFOX
	LF	1	TET
	LF	1	AMP, AMOX/CA
	LF		AMP, CHL
	LF	1	AMP, CFOX
	LF	1	AMOX/CA[I], CFOX
	LF	1	CFOX, STR
	LF	1	STR, TET
	LF	5	AMP, AMOX/CA, CFOX
	LF	1	AMP, AMOX/CA, CIPX[I]
	LF	1	CHL, STR, TET
	LF	1	AMOX/CA, CFOX, SMX/TMP[I], STR
	LF	1	AMOX/CA, CHL, STR, TET
	LF	3	AMP, CHL, KAN, STR, TET
	LF	1	AMP, AMOX/CA, CFOX, CHL, STR, TET
	LF	2	CFOX, CTAX, CTRX, KAN, TET, CIPX[I]
	LF	1	AMP, AMOX/CA, CTRX, CHL, SMX/TMP, STR, TET
	NLF	1	AMP, AMOX/CA, CFOX, CHL
	NLF	1	AMP, AMOX/CA, CFOX, CHL, SMX/TMP
	NLF	1	AMP, AMOX/CA, CFOX, CHL, SMX/TMP, TET
	NLF	1	AMP, AMOX/CA, CFOX, STR, TET
	NLF	1	AMP, AMOX/CA, CFOX, CHL, STR, TET
	NLF	1	AMP, AMOX/CA, CFOX, CTAX, CTRX, CHL, SMX/TMP, TET
	NLF	6	AMP, AMOX/CA, CFOX, CTAX, CTRX, GEN, KAN, IMIP, SMX/TMP[I], STR, TET
	NLF	2	AMP, AMOX/CA, CFOX, CIPX[I], CTAX, CTRX, GEN, KAN, IMIP, SMX/TMP, STR, TET

Table 5.3 continued

	Steno. maltophilia	1	AMP, AMOX/CA, CFOX, CTAX, CTRX, GEN, KAN, IMIP, STR, TET
	Steno. maltophilia	1	AMP, AMOX/CA, CIPX, CTAX, CTRX, CHL, GEN, KAN, IMIP, STR, TET
	Steno. maltophilia	1	AMP, AMOX/CA, CFOX, CIPX, CTAX, CTRX, CHL, GEN, KAN, IMIP, STR, TET
Past	LF	4	AMP
batch	LF	1	CHL
	LF	1	AMOX/CA[I]
	LF	8	AMP, CFOX
	LF	1	AMP, CHL
	LF	1	AMP, TET
	LF	1	AMP, CFOX, CHL
	LF	2	AMOX[I], CFOX, STR
	C. freundii	1	AMP, CFOX, STR, TET
1998			
Raw	NLF	1	AMP, AMOX/CA[I], STR
batch	*C.freundii*	1	AMOX/CA[I], CFOX
	C.freundii	2	AMP, AMOX/CA[I], CFOX
	C.freundii	1	AMP. CFOX, STR
	Ent. aerogenes	2	AMP, AMOX/CA[I], CFOX
	Ent. sakazakii	1	AMP, AMOX/CA
	Ent. sakazakii	1	AMP, AMOX/CA, CFOX
	Ent. sakazakii	1	AMP, CHL, GEN, KAN, STR
	E.coli	1	GEN
	E.coli	1	AMP, GEN, STR
	Hafnia alvei	1	AMP, AMOX/CA, CFOX
	Rahnella	1	AMP
	Rahnella	1	AMP, CFOX

Table 5.3 continued

	Ps.aeroginosa	1	AMP, AMOX/CA, CFOX, CHL, GEN, SMX/TMP
	Ps.aeroginosa	1	AMP, AMOX/CA, CFOX, CHL, GEN, KAN, SMX/TMP, STR
	Steno.maltophilia	1	AMP, AMOX/CA, CFOX, CTAX, CTRX, GEN, KAN, IMIP, STR
Past	NLF	1	AMP, AMOX/CA, CFOX, CHL, CTRX, GEN, KAN, STR
batch	*Acinetobacter*	1	AMOX/CA[I], CFOX, CHL, STR
	C.freundii	1	CFOX
	C.freundii	1	AMP, CFOX
	C.freundii	1	CFOX. STR
	Chromo. violaceum	2	AMP, AMOX/CA, CFOX, CHL, CTAX, CTRX, GEN, KAN, SMX/TMP[I], STR
	Chryseomonas	1	CFOX, CHL, STR
	Ent.gerganae	1	Fully susceptible
	Ent. sakazakii	3	AMP, AMOX/CA[I], CFOX
	Ent. sakazakii	1	AMOX/CA[I], CFOX, STR
	Ps. aeroginosa	1	AMP. AMOX/CA, CHL, CFOX, CTAX, CTRX
	Ps.aeroginosa	1	AMP, AMOX/CA, CFOX, CHL, CTRX, GEN, KAN, SMX/TMP[I], STR
	Serratia	1	AMP, AMOX/CA, CHL, CFOX, GEN

GENUS: *Steno.=Stenotophomonas; Ent.=Enterobacter; Ps.=Pseudomonas; C.=Citrobacter; E.=Escherichia; Chromo.=Chromobacter*

LF=Lactose fermenter. NLF=Non-lactose fermenter. Past=Pasteurised

Table 5.4 Summary of antibiotic resistance of gram negative bacteria isolated from milk

Strain type	No. strains	No. resistant to ≥ 3 antibiotics
1997		
LF	58	19
NLF	14	14
Citrobacter feundii	1	1
Steno. maltophilia	3	3
1998		
Enterobacter	10	2
E. coli	2	1
Pseudomonas	4	4
Steno. maltophilia	1	1
Citrobacter freundii	7	1
Serratia	1	1

Table 5.5. Resistance profiles of bacteria isolated from farm environments

Sample	Bacterial type	No. of strains	Antibiotic resistance profiles
1997			
Water	CNS	1	CFOX, ERY, PEN, RIF
Water	CNS	1	AMP, CFOX, ERY, PEN, RIF
Water	LF	1	AMP, CFOX
Water	LF	1	AMP, CFOX, STR
Water	LF	1	AMP, STR, TET
Feed	CNS	1	AMP, CFOX, CTAX, CTRX, ERY, KAN, STR
Feed	LF	1	CFOX
Feacal	CNS	1	AMP, CFOX, ERY, KAN, PEN, RIF, STR
Feacal	LF	1	CFOX, TET
1998			
Water	LF	1	AMP, AMOX/CA[I]
Water	LF	1	AMP, AMOX/CA, CFOX
Water	NLF	2	CHL
Water	*Aeromonas*	1	AMP, STR, IMIP[I]
Water	*Aeromonas sobria*	1	AMP, STR
Water	*Chryseomonas*	1	AMOX/CA[I], CFOX, CHL
Feed	NLF	1	AMP, AMOX/CA, CFOX
Feed	*Acinetobacter*	1	AMOX/CA[I], CFOX, CHL
Feed	*Enterobacter*	2	AMP
Feed	*Ent.skazakii*	1	AMP, STR. TET
Feed	*Klebsiella*	1	AMP
Feed	*Pseudomonas*	1	AMP, AMOX/CA[I], CHL, CFOX, CTRX, SMX/TMP, STR
Feed	*Serratia*	1	AMP
Feacal	*E.coli*	7	Fully susceptible
Feacal	*Kleb. oxytoca*	1	AMP
Feacal	*Pseudomonas*	1	Fully susceptible
Feacal	*Ps.fluorescens*	1	CFOX, SMX/TMP[I]

GENUS: *Ent.=Enterobacter; Ps.=Pseudomonas; C.=Citrobacter; E.=Escherichia; Kleb.=Klebsiella*

LF=lactose fermenter; NLF=non lactose fermenter; CNS=coagulase negative staphylococci

Table 5.6. Resistance profiles of gram negative bacteria isolated from vegetables

Species	No. of strains	Antibiotic resistance profiles
Acinetobacter	1	CFOX
	1	AMP, CFOX, CHL
C.freundii	1	AMP, KAN
	2	AMP, AMOX/CA, CFOX
Ent.agglomerans	3	Fully susceptible
	2	AMP
	1	AMP, AMOX/CA, CFOX
Ent. cloacae	1	AMP, AMOX/CA, CFOX
Ent.sakazakii	1	Fully susceptible
	2	AMP
	1	AMOX/CA, CFOX
	3	AMP, AMOX/CA, CFOX
	1	AMP, AMOX/CA, CFOX, CHL, SMX/TMP, STR
Ent. taylorae	1	AMP, AMOX/CA, CFOX
E. vulneris	2	Fully susceptible
Kleb.ozaneae	1	STR
Kluyvera spp	1	Fully susceptible
Lecl.adecarboxylata	4	Fully susceptible
	1	AMP, CFOX, CHL
Providencia	1	AMOX/CA
	1	AMP, AMOX/CA, CHL, SMX/TMP[I]
Pseudomonas	1	AMP, AMOX/CA[I], CFOX, CHL
	2	AMP, AMOX/CA, CFOX, CHL, SMX/TMP, STR,
Serratia	1	AMP
	1	AMOX/CA[I]
	1	AMP, AMOX/CA
	2	AMP, AMOX/CA, CFOX

GENUS: *Ent.=Enterobacter; C.=Citrobacter; E.=Escherichia; Kleb.=Klebsiella; Lecl.=Leclercia*

5.3 CONCLUSIONS

Staphylococci were resistant to early ß-lactam antibiotics by the 1960s (Wray, 1997). Methicillin and oxacillin, which are unaffected by the ß-lactamases of staphylococci, were introduced, but due to resistance via the acquisition of a gene encoding, a new PBP has now rendered all recent ß-lactams (conglomerate group classified as methicillin-resistant staphylococci) as ineffective. Resistance to gentamicin, the genes usually plasmid/transposon located, has also occurred. Ciprofloxacin resistance by chromosomal mutations has developed in many bacterial species. New quinolones such as trovafloxacin have been produced due to resistance to ciprofloxacin. In the present study, decreased susceptibility to ciprofloxacin was also noted.

Enterococci have a large number of acquired resistance genes and vancomycin is an important agent for hospital elimination of multi-resistant strains of *E. faecalis* and *E. faecium*. Although vancomycin resistance was not found in the enterococci studied, clinical strains have been isolated in Bloemfontein and environmental linkage has not necessarily been excluded in this limited study.

Enterobacter, *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas* species can present tremendous therapeutic problems as they acquire plasmid/transposon-mediated resistance to many agents. In the strains isolated from milk, multi-antibiotic resistance was evident and equivalent to that seen in hospital settings.

The use of antibiotics for veterinary medicine, prophylactics, growth promotion and feed preservation will certainly influence the prevalence of antibiotic-resistant bacteria that can enter the food chain. Antibiotic control policies are crucial in preventing the emergence and spread of resistance genes. The dilemma exists, however, that the community is unwilling to consume antibiotic-resistant bacteria. Neither is the intake of pathogenic bacteria in poor quality products recommended. The ideal would be products that are free from both antibiotic-resistant bacteria and pathogens.

CHAPTER 6

GENERAL DISCUSSION

The community and employees are adversely affected by consumption of and exposure to hazardous bacteria. A cause-related illness can be of a transient nature with self-limiting effects that can be serious, or even life-threatening or fatal. Bacteria may also have a latent phase, which makes diagnosis extremely difficult. It is imperative that food handlers and the community are protected by maintaining a high standard of control.

The highest possible quality of milk should reach the consumer. Not only microbiological counts, but the reliable detection of pathogens and antibiotic residue should also be part of routine analysis. It is of the utmost importance that no residual antibiotics should be detected in milk samples, and therefore individual cows must be tested on a regular basis after antibiotic administration.
Milk from cows with clinical or sub-clinical mastitis is often the cause of pathogenic organisms in milk. This is especially evident where parlour management is not up to standard.
Some of the milk samples from individual cows tested in this study were of a good hygienic quality. However, as soon as milk lots were combined in a batch, the quality declined remarkably. After effective pasteurisation at the farms, the milk quality normally conformed to standards. The majority of the milk sold at depots did not conform to one or more of the standards as specified by law and after pasteurisation contamination was found to be the main reason. Due to transportation with inadequate cooling, inadequate pasteurisation temperatures or contamination after pasteurisation, bacteria have the capacity to multiply.
The microbiological status of milk samples from all categories collected in the Bloemfontein area during 1997 and 1998 were generally considered unsatisfactory.

Enrichment culture media, where appropriate, were employed. The supplements contain a variety of enriching nutrients that were capable of supporting/promoting growth of desired bacteria or assist in the recovery of heat-stressed/injured cells that may be present in small numbers (Cappuccino & Sherman, 1996). Selective media were then used to isolate potentially pathogenic bacteria. Presumptive colonies were selected according to the manufacturers' protocol pertaining to the different selective media employed. Confirmation of a bacterial species was generally performed by means of the Gram-stain and using the appropriate API identification system.

In this study, apart from *S. aureus, Listeria* species and two ß-haemolytic streptococci, no other pathogens were detected. The *Listeria* isolation method is well-developed with two enrichment stages which emphasises the importance of the recovery of heat-stressed and injured cells. The improvement of other pathogen isolation techniques should be improved as it became clear in the study that colony morphology and in-agar biochemical testing are not satisfactory. Many potentially promising pathogens were eventually identified as opportunistic pathogens, such as *Enterobacter sakazakii* and *Ent. agglomerans*.

In the present study, identification of the majority of presumptive pathogens was performed by using API systems. The data bank for interpreting API profiles is based on clinical isolates and it might well be expected that bacteria from environmental sources may not totally conform.

Although the regulations state that food products for consumption should not contain any pathogens, regular laboratory testing for pathogens is not routinely performed throughout South Africa. The Dairy Regulations should mention specific pathogens, each with a reliable detection method, that can be used in routine laboratories.

The use of conventional microbiological methods in the detection and identification of pathogenic bacteria is time-consuming and expensive. Costs include: the selective media and different enrichment stages, the costs of the

laboratory workers, reagents and confirmation kits.

Education and training concerning the sanitation of equipment, the importance of the cold chain during distribution and personal hygiene are of the utmost importance for employees, as well as depot owners.

PCR is a very convenient method to identify organisms. Milk, however, is not an easy medium to incorporate into the PCR system. Difficulty was experienced in obtaining good reproducible target DNA, and the PCR product sizes were not always as expected. It will be necessary to test local strains and to continuously correlate and update PCR performance.

In this study it was found that certain factors in milk, not necessarily inhibitory substances, had an influence on the PCR results. It was found that freezing and thawing a milk sample adversely affected PCR results, and it is therefore advised that PCR should be done on fresh milk samples only. It is recommended that retrospective studies should not be conducted, unless the DNA preparation is totally free of milk components and can be treated as standard purified DNA.

The dynabead DNA concentration concept, which is vital for the detection of low cell numbers, performed very well, but was also affected by the freezing/thawing processes.

The conventional microbiological and PCR results concerning *L. monocytogenes* were in accordance. The use of PCR for the detection of *Campylobacter* and *Brucella* appeared promising. Problems were experienced with the *C. jejuni* stock cultures that proved to be non-viable. The two selected primer pairs for *B. abortus* appeared to be excellent, but, due to problems encountered during the study, their full value could not be evaluated.

Even under the strictest of controlled conditions and in the most competent hands, PCR can inexplicably go astray. Once a problem becomes evident, it is extremely difficult to locate the source, and it is very time consuming to exclude.

Although PCR technology can be fraught with inherent problems, it remains the most comprehensive and convenient option for future rapid detection/

identification of foodborne pathogens. Further research on PCR application for milk sample testing should continue in order to standardise.

Problems experienced with antibiotic administration for a variety of reasons have lead to the misuse of antibiotics by farmers. Lack of knowledge and the use of growth promoters could be a reason for the incidence of antibiotic-resistant strains in the environment (Chapter 5). The incidence of increased antibiotic-resistant strains, in clinical situations and in the environment, is reason for concern.

The resistance profiles of staphylococci isolated from raw and pasteurised milk during 1997 and 1998 showed *S. aureus* resistance to three or more antibiotics with streptomycin, tetracycline and cephalosporin resistance evident. The majority of antibiotic-resistant CNS carried resistance to ciprofloxacin, streptomycin and disturbingly oxacillin.

Veterinarians will probably have major problems trying to successfully treat gram-negative bacterial mastitis. Ampicillin and augmentin, which is still used by general practitioners for human infection, are likely to be ineffective. Several NLF strains isolated in 1997 were resistant to eight or more antibiotics.

Milk is one of the most nutritious products available at an affordable price. In a country where the majority of the community lives in poverty, it is a product of critical importance. Consumers have the right to receive milk of good quality, which is free from pathogens and antibiotic residue.

It is suggested that databanks for antibiotic resistance profiles of strains in milk, vegetable produce and the environment should be designed. Furthermore, it is recommended that education programmes should be established for farmers and other people involved in the food industry to emphasise the prevalence and implications of antibiotic resistance.

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APPENDIX

1. CONVENTIONAL METHODS

1.1. Milk

Table A.1 Non-microbiological test compiled on milk

No	Phosphatase	Stability	Inhibitors
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-
8	-	-	-
9	+	-	-
10	+	-	-
11	+	-	-
12	+	-	-
13	+	-	-
14	+	-	-
15	+	-	-
16	+	-	-
17	+	-	-
18	+	-	-
19	+	-	-
20	+	-	-
21	+	-	-
22	-	-	-
23	-	-	-
24	+	-	-
25	+	-	-
26	+	-	-
27	+	-	-
28	-	-	-
29	+	-	-
30	+	+	-
31	+	-	-
32	+	+	-
33	-	-	-
34	+	-	-
35	+	-	-

Table A.1 continued

36	-	-	-
37	-	-	-
38	+	-	-
39	+	-	-
40	-	-	-
41	+	-	-
42	+	-	-
43	+	-	-
44	+	-	-
45	-	-	-
46	+	-	-
47	+	-	-
48	+	-	-
49	+	-	-
50	-	-	-
51	-	-	-
52	-	-	-
53	+	-	-
54	-	-	-
55	+	-	-

Table A.2 Presumptive pathogenic organisms isolated from milk

No	*S. aureus*	CNS	Presumptive pathogenic strains isolated
1	-	-	-
2	-	-	-
3	-	-	*Proteus vulgaris*
4	-	-	*E. vulnneris, Hafnia halvei*
5	-	-	-
6	+	-	-
7	+	-	*E. hermannii*
8	-	-	-
9	+	-	-
10	+	+	*Ent. agglomerans*
11	-	+	-
12	-	-	-
13	+	-	*Ent. agglomerans*
14	-	-	-
15	-	-	-
16	-	+	*Lecl. adecarboxylata, List. monocytogenes*
17	-	-	-
18	-	+	*C. freundii, List.monocytogenes*

Table A.2 continued

19	+	+	-
20	+	+	-
21	-	+	-
22	-	-	-
23	-	+	*Providencia*
24	+	+	*Providencia*
25	-	-	-
26	+	+	-
27	+	+	*Steno. maltophilia*
28	-	-	-
29	+	+	Providencia, *Steno. maltophilia*
30	+	-	*Pseudomonas, Steno. maltophilia*
31	-	-	*Pseudomonas, Steno. maltophilia*
32	+	-	*Steno. Maltophilia*
33	-	-	-
34	+	+	*Aer. Hydrophila*
35	-	-	*C. freundii*
36	-	-	*C. freundii, List. monocytogenes*
37	-	-	-
38	+	-	-
39	+	-	-
40	-	+	*Aer.hydrophila*
41	-	-	-
42	-	-	-
43	-	-	*Ent. agglomerans*
44	+	+	*List. monocytogenes*
45	-	-	-
46	+	+	-
47	+	+	-
48	-	-	*E. coli*
49	-	+	-
50	-	+	*Ent. gergoviae*
51	+	+	*C. freundii*
52	-	+	*C. freundii*
53	+	+	*Serratia marcescens, Rahnella aquatilis*
54	-	-	-
55	+	+	*Hafnia halvei, Ent. sakazakii, Rahnella*

1.2. Vegetables:

Table A.3 Presumptive pathogenic colonies isolated from vegetable samples

Vegetable	no	B.c	Yers	Kligler	Clos	McC	SS	List.Fraser	List.agar
Cabbage L	1	+	-	-	-	-	-	-	
Cabbage C	2	-	-	-	-	-	-	+	-
Carrots L	3	-	+	+	-	-	+	+	-
Carrots C	4	-	+	-	-	-	+	+	-
Lettuce L	5	-	-	-	-	-	+	+	-
Lettuce C	6	-	-	-	-	-	-	-	
Mushroom L	7	-	-	-	-	-	-	+	-
Mushroom C	8	+	-	-	-	-	-	+	+
Spinach L	9	-	+	+	-	-	-	+	-
Spinach C	10	-	-	-	-	+	-	+	+
Beans L	11	-	-	-	-	-	-	+	+
Broccoli C	12	-	-	-	-	-	+	+	-
Cabbage L	13	+	+	+	-	-	-	-	
Lettuce L	14	-	+	+	-	-	+	+	-
Beans L	15	+	+	+	-	+	+	-	
Carrots L	16	-	+	-	-	-	+	+	-
Carrots C	17	-	+	-	-	+	-	+	-
Broccoli C	18	-	+	-	-	+	-	+	-
Mushroom C	19	-	+	-	-	+	-	+	-
Celery C	20	-	+	+	-	+	+	+	-
Lettuce C	21	+	+	-	-	-	-	+	+
Cabbage C	22	-	+	+	-	-	-	-	
Spinach C	23	+	-	-	-	-	-	+	-
Lettuce C	24	-	+	+	-	-	-	-	
Cabbage L	25	-	-	-	-	-	-	+	-
Broccoli C	26	-	-	-	-	-	-	-	
Cucumber C	27	-	+	+	-	-	-	+	-
Mushroom C	28	-	+	-	-	-	-	+	-
Spinach C	29	-	+	-	-	-	-	+	+
Carrots C	30	+	+	+	-	-	-	-	
Carrots L	31	-	+	-	-	-	-	-	
Mushroom L	32	-	-	-	-	-	-	+	-
Beans L	33	-	+	-	-	-	-	+	+
Tomato C	34	-	-	-	-	-	-	-	
Broccoli C	35	-	-	-	-	-	-	+	-
Mushroom C	36	-	-	-	-	-	-	+	-
Carrots C	37	-	-	-	-	-	-	+	-
Lettuce C	38	-	+	-	-	-	-	+	-
Cabbage C	39		+	-	-	-	-	+	-

Table A.3 continued

Cabbage L	40	-	+	-	-	-	-	+	-
Spinach L	41	-	-	-	-	-	-	+	-
Beans L	42	-	+	-	-	-	-	+	-
Carrots L	43	-	+	-	-	-	-	+	-
Mushroom L	44	-	+	-	-	-	-	+	-
Lettuce L	45	-	+	-	-	-	-	+	-

2. PCR RESULTS

Table A.4 PCR on lysed *Listeria* cultures and viability of inoculated cells

Time	Treatment	No	Viable count	PCR Treatment	Bands	
					U	LM
Day 1	Inoculated to $2.5x10^4$ cfu/ml in milk	1	1288	Dynabead	-	+
	Pasteurised at 63°C for 30 minutes	2	80	Dynabead	+	+
Day 3	Past, kept at 4°C	3	Tntc	Dynabead	-	+
	Past, on bench	4	Tntc	Dynabead	+	-
	Repasteurised, 65°C for 90 minutes	5A	0	Dynabead	+	+
		5B		1.5µl direct	-	-
Day 7	Repast, kept at 4°C	6	0	Dynabead	+	+
	Repast, on bench	7	tntc	Dynabead	+	+
	Control lysate	8		Direct	+	-
	16 lysate	9		Direct	-	-
	18 lysate	10		Direct	-	-
	36 lysate	11		Direct	+	+
	44 lysate	12		Direct	+	+

Table A.5 *Listeria* primer results

DATE	SAMPLE	DYNABEADS	U1,2 PRIMERS	LM1,2 PRIMERS
05/08	Milk 16	+	+	+
	Milk 18	+	+	-
	Milk 36	+	+	-
	Milk 44	+	-	-
	Milk 16	-	+	+
	Milk 18	-	+	-
	Milk 36	-	+	-
	Milk 44	-	+	+
	Control	+	+	-
12/08	Lysate 16	+	-	-
	Lysate 18	+	-	-
	Lysate 36	+	+	+ (700)
	Lysate 44	+	+	+ (700)
06/08	Milk 19	+	+	+
	Milk 20	+	+	+
	Milk 21	+	+	-
	Milk 29	+	-	-

Table A.6 Effect of sample treatment on PCR

Treatment	Number	PCR Treatment	Bands U	LM
Milk 16	1	Dynabead	-	-
Milk 18	2	Dynabead	-	-
Milk 36	3	Dynabead	-	-
Milk 44	4	Dynabead	-	-
Lysate 16	5	1.5µl direct	-	-
Lysate 18	6	1.5µl direct	-	-
Lysate 36	7	1.5µl direct	-	-
Lysate 44	8	1.5µl direct	-	-
Lysate Control	9	1.5µl direct	-	-
Inoculated, on bench	10	Dynabead	+	+
Inoculated, at 4°C	11	Dynabead	+	+
Inoculated, on bench	12	Dynabead	-	+
Inoculated, at 4°C	13	Dynabead	-	+
Past, at 4°C	14	Dynabead	-	+
Past, on bench	15	Dynabead	-	+
Repast, at 4°C	16	Dynabead	-	+
Repast, on bench	17	Dynabead	+	+
Control lysate	18	5µl + Dynabead	-	+

Table A.7 Summary of results obtained with different treatments and primer combination

Date	Sample	Dynabeads	U1/2 primers	LM1/2 primers
05/08	Milk 16	+	+	+
	Milk 18	+	+	-
	Milk 36	+	+	-
	Milk 44	+	-	-
	Milk 16	-	+	+
	Milk 18	-	+	-
	Milk 36	-	+	-
	Milk 44	-	+	+
	Control	+	+	-
12/08	Lysate 16	+	-	-
	Lysate 18	+	-	-
	Lysate 36	+	+	+ (700)
	Lysate 44	+	+	+ (700)
06/08	Milk 19	+	+	+
	Milk 20	+	+	+
	Milk 21	+	+	-
	Milk 29	+	-	-
	Milk 30	+	+	-
	Milk 32	+	+	+
	Milk 34	+	-	-
	Milk 35	+	+	+
	Milk 37	+	+	-
	Milk 39	+	-	-
	Milk 40	+	+	-
	Milk 41	+	+	-
	Milk 43	+	-	-

12/08	Innoculated milk			
	Unpast day1	+	-	+
	Past	+	+	+
	Past 4° day3	+	-	+
	Past bench	+	+	-
	Repast	+	+	+
	Repast	Direct	-	-
	Repast 4° day 7	+	+	+
	Repast bench	+	+	+ (700)
19/08	4° day3	+	+	+
	Bench	+	+	+
	4° day7	+	-	+
	Bench	+	-	-
	Past 4°	+	-	+
	Past bench	+	-	+
	Repast 4° day11	+	-	+
	Repast bench	+	+	+

Table A.8. Primer combinations and band size

Treatment	Primer combination			
	U1LM2	LM1U2	LM1&2	U1&2
Unpast, day 1	-	-	-	+
Past, day1	-	-	+(700)	+
Repast, day 7	-	-	+(700)	+
Repast, day 11	-	-	+(700)	-
Past, kept at 4°C, day7	-	-	+(700)	-
Milk 29			+(600)	+
Milk 43			+(600)	+

12/08	Innoculated milk			
	Unpast day1	+	-	+
	Past	+	+	+
	Past 4° day3	+	-	+
	Past bench	+	+	-
	Repast	+	+	+
	Repast	Direct	-	-
	Repast 4° day 7	+	+	+
	Repast bench	+	+	+ (700)
19/08	4° day3	+	+	+
	Bench	+	+	+
	4° day7	+	-	+
	Bench	+	-	-
	Past 4°	+	-	+
	Past bench	+	-	+
	Repast 4° day11	+	-	+
	Repast bench	+	+	+

Table A.8. Primer combinations and band size

Treatment	Primer combination			
	U1LM2	LM1U2	LM1&2	U1&2
Unpast, day 1	-	-	-	+
Past, day1	-	-	+(700)	+
Repast, day 7	-	-	+(700)	+
Repast, day 11	-	-	+(700)	-
Past, kept at 4°C, day7	-	-	+(700)	-
Milk 29			+(600)	+
Milk 43			+(600)	+

Table A.9. Direct application of PCR on milk for Listeria detection

Milk sample no	PCR mix with U1&2 and LM1&2	Milk	U1&2
9		9 raw	+
10	NO BANDS	10 raw	
11		11 raw	
12	REDO WITH	19 raw	
13	UNIVERSAL AND	20 raw	
15	LISTERIA	21 raw	
16	PRIMERS APART	29 raw	
17		30 raw	
18	NEW REAGENTS	32 raw	
19		39 raw	
20	NO BANDS	41 raw	
21		43 raw	
24		46 raw	
25		47 raw	
26		48 raw	
27		49 raw	
29		53 raw	
30		55 raw	
31		34 past	
32		35 past	
36		37 past	
39		40 past	+
41		50 past	
42		51 past	
43		52 past	
44		54 past	

Table A.10. Milk samples had been spiked with the MWM in order to find out if the milk had any DNase activity.

Milk	Treatment	Bands
19	Dynabead, 3µl MWM+3µl sample	+
46	Dynabead, 3µl MWM+3µl sample	+
10	3µl MWM+3µl sample	+
19	3µl MWM+3µl sample	+
46	3µl MWM+3µl sample	+
47	3µl MWM+3µl sample	+
UHT	3µl MWM+3µl sample	+

Table A.11. The effect of freezing and thawing on PCR detection

Sample	Treatment	Band with U1&2
Milk 1	200µl spun for 5min at 7000rpm	+
Milk 2	Supernatant removed and	-
Milk 3	Dynabeads add	-
Milk 1	200µl spun for 5min at 7000rpm	+
Milk 2	Supernatant removed and 25µl	-
Milk 3	99°C 10min and 1.5µl PCR	+
Milk 1 3x frozen	200µl spun for 5min at 7000rpm	-
Milk 2 1x frozen	Supernatant removed and	-
Milk 3 1x frozen	Dynabeads add	-
Milk 1 Dynabeaded 1x frozen		+ very light
Milk 2 Dynabeaded 1x frozen		-
Milk 3 Dynabeaded 1x frozen		-
Milk 1 4x frozen	200µl spun for 5min at 7000rpm	-
Milk 2 2x frozen	Supernatant removed and 25µl	-
Milk 3 2x frozen	99°C 10min and 1.5µl PCR	-

Table A.12. Total *Listeria monocytogenes* counts in milk on Listeria selective agar after different procedures

Milk treatment	Total *Listeria* count/ml
Inoculated milk with ±1250 cfu/ml, 0 h	1288
Pasteurised 63°C for 30 min, 0 h	80
Inoculated milk, 4°C, 24 h	tntc
Inoculated milk, 25°C, 24 h	tntc
Past. milk, 4°C, 24 h *a	800
Past. milk, 25°C, 24 h *b	tntc
Repasteurised *a at 63°C for 90 min, 0 h	0
Repasteurised *b at 63°C for 90 min, 0 h	0
Repasteurised 4°C, 72 h	0
Repasteurised 25°C, 72 h	tntc

Tntc = too numerous to count

3. ANTIBIOTICS

3.1 Stock Solutions

Sterile distilled water

95% Ethanol

0.1 M Phosphate buffer, pH 6 (autoclave)
11.9g KH_2PO_4 + 2.2g K_2HPO_4

0.1 M Phosphate buffer, pH 8 (autoclave)
0.9g KH_2PO_4 + 16.3g K_2HPO_4

2.5 M NaOH
100g NaOH/l distilled water (autoclave)

0.05 M HCl
4.2ml concentrated HCl added to 995.8 ml distilled water (autoclave)

Table A.13 Antibiotic stock solutions

Antibiotic	Conc.	Solvent	Diluent	Break point µg/ml
Ampicillin	98%	Phosphate buffer pH8	Phosphate buffer pH6	16
Penicillin G	100%	Water	Water	0.25
Cefoxitin	99.4	Water	Water	16
Cefotaxime	95%	Water	Water	16-32
Ceftriaxone	100%	Water	Water	16
Erythromycin	98%	Water	Water	1
Tetracycline	95%	Water	Water	8
Rifampicin	95%	Methanol	Water	2
Streptomycin	765 units	Water	Water	8
Gentamycin	647µg/mg	Water	Water	8
Vancomycin	1099 units	Water	Water	8
Kanamycin	784µg/mg	Water	Water	32
Chloramphenicol	100%	95% Ethanol	water	16