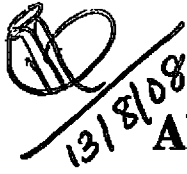
172312



13/8/08

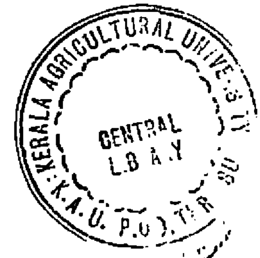
APPLICATION OF POLYMERASE CHAIN REACTION FOR RAPID EVALUATION OF HYGIENIC STATUS OF MILK

DEEPA MARY. J. J.

Thesis submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur



KERALA AGRICULTURAL UNIVERSITY CENTRAL LIBRARY K.A.U. P.O. THRISSUR

2008

Department of Veterinary Public Health
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

DECLARATION

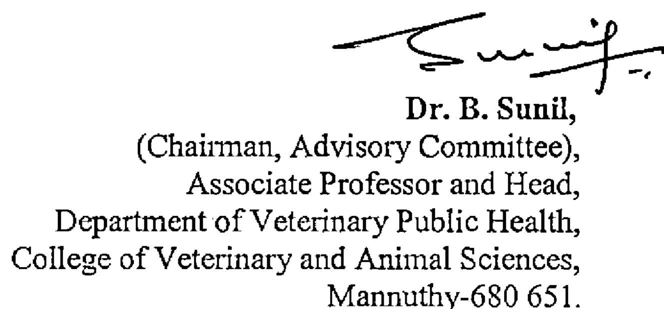
I hereby declare that this thesis entitled **"APPLICATION OF POLYMERASE CHAIN REACTION FOR RAPID EVALUATION OF HYGIENIC STATUS OF MILK"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Mannuthy
13/8/2008

DEEPA MARY J. J.

CERTIFICATE

Certified that this thesis, entitled **"APPLICATION OF POLYMERASE CHAIN REACTION FOR RAPID EVALUATION OF HYGIENIC STATUS OF MILK"** is a record of research work done independently by **DEEPA MARY J. J.**, under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

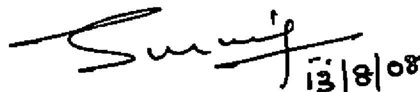


Mannuthy
13 · 8 · 2008

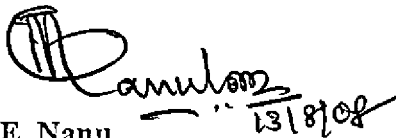
Dr. B. Sunil,
(Chairman, Advisory Committee),
Associate Professor and Head,
Department of Veterinary Public Health,
College of Veterinary and Animal Sciences,
Mannuthy-680 651.

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Deepa Mary J. J.**, a candidate for the degree of Master of Veterinary Science in Veterinary Public Health, agree that the thesis entitled **"APPLICATION OF POLYMERASE CHAIN REACTION FOR RAPID EVALUATION OF HYGIENIC STATUS OF MILK"** may be submitted by **Deepa Mary J. J.**, in partial fulfilment of the requirement for the degree.

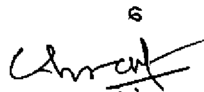


Dr. B. Sunil,
(Chairman, Advisory Committee),
Associate Professor and Head,
Department of Veterinary Public Health,
College of Veterinary and Animal Sciences,
Mannuthy-680651



Dr. E. Nanu,
Dean,
(Member, Advisory Committee)
College of Veterinary and Animal Sciences, Mannuthy-680 651.

Dr. P. Kuttinarayanan
(Member, Advisory Committee)
Professor and Head,
Department of Livestock Products Technology,
College of Veterinary and Animal Sciences, Mannuthy-680 651



Dr. T. V. Aravindakshan,
(Member, Advisory Committee)
Professor,
Centre for Advanced Studies in Animal Genetics and Breeding,
College of Veterinary and Animal Sciences, Mannuthy-680 651

External Examiner 04/11/08
(Dr. M. Sathyanarayana Rao)

ACKNOWLEDGEMENT

I express my sincere thanks to Dr. B. Sunil, Associate Professor and Head, Department of Veterinary Public Health, for providing a very interesting topic, full freedom, and remaining a fountain head of normal support throughout. I gratefully acknowledge his highly perceptive mind in planning and guiding the research work, suggestions and generous contribution of time and valid thoughts which helped me immeasurably to complete this work.

I deem it my privilege in expressing my reverence and thankfulness to Dr. E. Nanu, Dean, College of Veterinary and Animal Sciences, Mannuthy also a member of my advisory committee for his guidance, affection, wholehearted cooperation and the facilities provided for this research work.

I gratefully acknowledge Dr. P. Kuttinarayanan, Professor and Head, Department of Livestock Products Technology, for the incessant encouragement, affection and advice rendered to me as a member of my advisory committee.

I am cordially indebted to Dr. T. V. Aravindakshan, Professor, Department of Animal Breeding and Genetics for the supporting attitude, professional guidance and pleasant cooperation rendered to me during the entire period of research work.

I owe my sincere thanks to Dr. Reghu Ravindran, Assistant Professor, Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pookot, for his timely help and valuable suggestions.

I am indebted to Mr. P. R. Chandrasekaran, Instrumentation Engineer, for his eagerness to help at all times. The dedication shown by him in the upkeep of instruments is indeed commendable.

Special thanks to Mr. A. M. Binoy, Senior Research Fellow, Centre for Advanced Studies in Animal Breeding and Genetics for his valuable suggestions and timely help which made the task easier.

I am in short of words to express my deep sense of gratitude to Dr. Asha, Research Associate, Dr. Magna, Senior Research Fellow, and Dr. Prejit former Research Associate in the Department of Veterinary Public Health.

I greatly obliged for the facilities and financial help offered by ***Indian Council of Agricultural Research****, as the study was a part of Ad-hoc ICAR project entitled "Evaluation of quality of market milk with special reference to quality assurance programme in Kerala" implemented through Department of Veterinary Public Health.*

I would like to place on record my heartfelt thanks to Dr. Satyanarayana Rao for the encouraging advice.

I warmly remember the cooperation rendered by other teachers Dr. Vrinda Menon and Dr. Sethulekshmi C. in the Department of Veterinary Public Health, during the final stages of the thesis preparation.

I cherish the spirit of understanding and personal encouragement rendered to me by my friends Drs. Bhagyalakshmi, Remya, Ambili, Geetha, Manu. and Divya

I gratefully acknowledge the help rendered to me by my friends Drs. Jimcy, Resmi, Indu, Bincy, Albert, Midhun, Rojan, Jini, and Anupama.

I sincerely thank my dear friends Drs. Savitha, Nimmy, Bhadra and Chandini K.G. for the help, support and friendship extended by them.

I warmly remember and acknowledge the keen interest shown, help and support rendered to me by my friends Arun and Simi through the entire period of research work.

I am in short of words to express my thanks for the invaluable help, support, and affection from my dear friend Dr. Nimila.

I wish to extend my thanks to the head and other employees of the Livestock Farm, KAU, who helped me, collect the samples.

I remember with gratitude the free supply of a costly reagent by Mr. Andreas Steinhoss of Bioron, Germany.

I am thankful to Mr. Achuthanandan, Ms. Bindu, Ms. Sheela, Ms Anitha and Ms. Sumathi for the cooperation rendered to me during the study.

No words can express my gratitude to my beloved parents, for their constant encouragement and prayers which gave me the confidence to continue the work and complete it successfully.

Above all I bow before God, the Almighty for all the blessings showered upon me for the successful completion of this work. To Him, I dedicate this work with reverence.

CONTENTS

Chapter	Title	Page No.
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	4
3	MATERIALS AND METHODS	36
4	RESULTS	52
5	DISCUSSION	62
6	SUMMARY	69
	REFERENCES	71
	ABSTRACT	

LIST OF ILLUSTRATIONS

LIST OF TABLES

Table No.	Title	Page No.
1	Comparison of time taken by different DNA extraction procedures	57
2	Detection level of pathogens by PCR in PBS	58
3	Detection level of pathogens by PCR in milk	58
4	Estimation of bacterial load by PCR and culture method	59
5	Comparative assessment of detection of *E. coli* in milk by PCR and conventional culture method	60
6	Comparative assessment of detection of *S. aureus* in milk by PCR and conventional culture method	61

LIST OF FIGURES

Figure No.	Title	Between Pages
1	*Pseudomonas aeruginosa*	53&54
2	PCR of *E.coli* using template DNA extracted using various temperatures of incubation	54&55
3	Sensitivity of PCR for Pseudomonas species	56&57
4	Sensitivity of PCR for *E. coli*	56&57
5	Sensitivity of PCR for *Staphylococcus aureus*	56&57
6	Assessment of bacterial load in milk based on Pseudomonas species	59&60

LIST OF PLATES

Plate No.	Title	Between Pages
1	*Escherichia coli* in Hicrome E.coli Agar	60&61
2	Staphylococcus aureus in Baird-Parker Agar	60&61

LIST OF FLOW CHARTS

Flow Chart No:	Title	Between Pages
1	Preparation of template DNA of *Pseudomonas aeruginosa/ Escherichia coli* from culture (Boiling lysis)	40&41
2	Preparation of template DNA from pure culture of gram negative bacteria (*Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus*) by phenol chloroform extraction (Phuektes *et al.* 2001)	41&42
3	Extraction of DNA extraction from milk	41&42

Introduction

1. INTRODUCTION

The microbiological quality of milk reveals the hygienic status of production, the keeping quality, fitness for further processing and presence of pathogens in milk. The conventional culture based methods for evaluating the bacterial load in milk involves a time of around 24-48 hours and a few days for isolation and identification of the organism. In the dairy industry there is a need for rapid and reliable tool to assess the bacterial load in as short a period as possible. Rapid detection of microorganisms including pathogens would allow rejection of inferior quality milk, monitoring of process contamination and provide a more accurate estimation of shelf life of the finished products.

Several rapid automated methods have been developed to overcome the inherent disadvantages in the enumeration of bacteria in milk (Gonzalez *et al.* 1994). Polymerase chain reaction based assay has been developed for rapid screening of pathogenic microorganisms in food. The simplicity of this technology together with its potential for detecting small number of target organisms without a need for culturing of cells makes it an important tool for monitoring hygiene and pathogens in food (Bej *et al.* 1990).

Food safety is a major public health concern as contaminated food may contain high risk pathogens that cause diseases resulting in loss of time and money. Milk being a natural perfect food which is consumed widely, has to be handled carefully during production, processing, marketing till consumed as it can become a major source of pathogens and food poisoning outbreaks. This problem is of tremendous public health importance in a country like India where production and handling of milk are below the satisfactory level. India leading in milk production (provided by the millions of small marginal farmers) with one third of the world bovine population indicates low productivity per animal. Milk a favorable medium for the growth of

microbes, obtained from scattered sources, may be consumed raw, a major part of the diet of infants and children for want of hygiene can pose a threat to public health.

Despite major technological advancements in milk processing industry, incidents of foodborne diseases associated with dairy industry continue to occur across the world, necessitating greater diligence in the control of pathogens. Detection, identification and quantification of food borne pathogens are often made difficult due to the problems associated with determining whether an outbreak has occurred, the difficulty in recovering the agents responsible for the food borne outbreaks due to low number of pathogenic bacteria among the high number of contaminating and indigenous bacteria and interference from food matrix and the lack of methods powerful enough to detect specific food borne pathogens.

Prior to the advent of molecular biology techniques, identification of microbial pathogens relied on bacteriological methods to isolate the organism from clinical and food samples and subsequent confirmation of microbes identity by biochemical or immunological methods. Recent developments in techniques has suggested that DNA based tests will be faster, more sensitive and more specific for monitoring microbial contaminants. When Polymerase chain reaction was first described in 1985, the field of DNA analysis changed dramatically. The ability to rapidly obtain large quantities of a defined region of DNA from a few original copies has led to reassessment of many old procedures, and so a host of new applications. Polymerase chain reaction offers a new level of sensitivity and speed for the detection of food borne pathogens, reducing the lengthy enrichment and isolation of microorganisms.

Many of the foods with short shelf life are consumed by the time the results of typical microbial analysis are completed. The application of various quality assurance programmes in milk, industry like the HACCP requires a rapid assessment of bacterial load as well as detection of pathogens of public health significance in milk.

There is a need for more rapid sensitive and specific methods that will facilitate the identification of contaminated food products.

Hence the present study was taken up with the following objectives.

1) Develop a rapid Polymerase Chain Reaction (PCR) based methodology to evaluate the hygienic status of milk within eight hours from the collection of samples.

2) Application of PCR for rapid detection of *Escherichia coli* and *Staphylococcus aureus* from milk.

Review of Literature

2. REVIEW OF LITERATURE

2.1 AEROBIC PLATE COUNT / BACTERIAL LOAD

Aerobic Plate Count (APC) is an indicator of bacterial population in a sample. The test is based on the assumption that each organism will form a visible colony when mixed with agar containing the appropriate nutrients. It does not measure the entire bacterial population, but the organisms that grow aerobically at mesophilic temperatures. Aerobic plate count can give the food processor information regarding raw materials, processing conditions and handling of product. Aerobic plate count with pour plate technique requires 48 h incubation. So the result is available only after two days.

The number of psychrotrophs generally required to initiate spoilage in milk is about 10^6 colony forming units per milliliter (cfu/ml) (Birkeland *et al.*, 1985).

Eneroth *et al.* (2000), found that pasteurized milk can get contaminated during automatic filling of retail packages.

2.1.1 Conventional culture method

Latha and Nanu (1997) investigated the bacterial quality of 12 samples each of pasteurized milk obtained from five different sources. The mean aerobic plate count of the samples were 5.98 ± 0.05, 5.23 ± 0.06, 5.57 ± 0.06, 4.58 ± 0.03 and 3.55 ± 0.01 $\log_{10}$ cfu/ml.

Jolly *et al.* (2000) evaluated 60 raw market milk collected from three sources located in and around Mannuthy. From each source 10 each of pooled and individual milk samples were collected. The mean total viable count of individual milk samples were 5.93 ± 0.05, 6.12 ± 0.23 and 6.2 ± 0.12 $\log_{10}$ cfu/ml and the mean total viable

count of pooled milk samples obtained from the sources were 6.06 ± 0.11, 6.78 ± 0.26 and 6.04 ± 0.10 $\log_{10}$ cfu/ml.

Sethulekshmi *et al.* (2003) assessed the bacterial quality of 84 samples of toned pasteurized milk retailed in and around Thrissur. The overall mean total viable count of the samples was 2.82 ± 0.14 $\log_{10}$ cfu/ml.

Chye *et al.* (2004) determined the microbiological safety of raw milk from 360 dairy farms in Peninsular Malaysia. Milk samples were collected at 40 milk collection centers from four regions, namely, Southern (Johor/Melaka), Central (Selangor/Negeri Sembilan), Northern (Perak/Kedah) and Eastern (Kelantan/Terengganu) according to stratified random sampling design. Samples were analyzed for Total Plate Count, *Staphylococcus aureus,* coliforms and *Escherichia coli* as well as the prevalence of selected pathogens such as *Listeria monocytogenes*, *E. coli* O157:H7 and Salmonella. From the 930 milk samples tested, approximately 90 per cent were contaminated by coliform bacteria and 65 per cent were *E. coli* positive, with mean counts ranged from 10^3 to 10^4 cfu/ml. *S. aureus* was isolated from more than 60 per cent of the samples and the mean count per ml was 12 x 10^3. Meanwhile, *E. coli* 0157:H7 was also detected in 312 (33.5 per cent) samples. However, Salmonella was only detected in 1.4 per cent of the samples.

Prejit (2005) investigated the bacteriological quality of 60 raw milk samples which included 20 each from individual animals and pooled raw milk from University Livestock Farm, Kerala Agricultural University and 20 chilled raw milk samples from Kerala Agricultural University Dairy Plant. The average total viable count of milk from individual animals, pooled milk and chilled milk samples were 5.14 ± 0.13, 5.58 ± 0.14 and 5.70 ± 0.13 $\log_{10}$ cfu/ml respectively.

Jaibi (2006) analyzed the microbial quality of a total of 144 raw milk samples, consisting of individual and pooled milk samples collected from three societies. The

overall mean total viable count of individual samples was 6.12 ± 0.07 log_{10} cfu/ml and that of pooled milk samples was 6.52 ± 0.08 log_{10} cfu/ml.

Lekha (2006) assessed the bacterial quality of milk at the point of production from three societies in Kerala and found that the highest total viable count was at the level of 10^6 cfu/ml and the lowest was at the level of 10^4 cfu/ml.

Asha (2007) reported that the total viable count of pooled raw milk samples from two dairy plants in Thrissur were of the order of 10^7cfu/ml which was attributed to the lack of hygienic measures observed in the plant.

Gini (2007) evaluated the total viable count of milk in three societies in Thrissur and found the highest count at the level 10^7 cfu/ml out of 108 samples, 10^6 cfu/ml in 44 samples, 10^5 cfu/ml in 42 samples and 10 samples had 10^4cfu/ml.

Raw milk samples were collected from various local milk collection centers in different parts of Kanchipuram district, South India to enumerate and to identify bacteria with proteolytic and lipolytic activity. The total bacterial count of 26.7 per cent exceeded the hygienic limit in raw milk. (Prakash *et al.*, 2007).

2.1.2 PCR and related methods

May *et al.* (1989) suggested that Limulus Amoebocyte Lysate (LAL) assay procedure which required only 1.5 h would provide rapid information on the bacteriological quality of raw and finished products. The study showed that LAL assay specific for gram negative bacteria- lipopolysaccharide is proportional to the psychotropic bacterial count (most causes of spoilage during refrigeration) and aerobic plate count.

Hill (1996) suggested that it would be better to take food samples for template preparation at two different time intervals to avoid false positive results. If the cells

are growing, the PCR product yield from the second sample would be much greater because of increase in the amount of template DNA.

Vaitilingom *et al.* (1998) applied reverse transcriptase polymerase chain reaction (RT-PCR) to a mix of live and dead cells after heat treatment of *E. coli, S. cerevisiae* and *M. racemosus* in contaminated milk. The RT-PCR was negative when all cells were dead. With increased number of viable cells gel electrophoresis showed a quantitative increase in the intensity of specific band. The detection limit was 10 cells per millilitre of milk.

Li and Drake (2001) compared the cell densities from plate counts and Quantitative Competitive PCR (QC-PCR). Correlation analysis and regression analysis were conducted to determine the ability of QC-PCR to predict viable plate counts. At lower cell densities the predicted number by QC-PCR was low, possibly due to the decrease in DNA extraction efficiency at low cell numbers.

Martins *et al.* (2005) verified the presence of *apr* gene which encodes for alkaline metalloprotease in Pseudomonas and other related bacteria in psychrotrophic proteolytic bacteria isolated from raw milk collected from cooling tanks. A polymerase chain reaction (PCR) technique was used with degenerate primers. Total DNA from 112 isolates was pooled in different groups and then used as template for the amplification reactions. Controls consisted of DNA extracted from 26 cultures. An expected DNA fragment of 194 bp was detected in groups that contained bacteria identified as Pseudomonas. The PCR product was observed only when DNA from control cultures of *Pseudomonas aeruginosa, Pseudomonas fluorescens, Serratia marcescens and Aeromonas hydrophila* were used. A detection limit assay indicated that the *apr* gene could be directly amplified from pasteurized milk inoculated with 10^8 cfu/ml of *P. fluorescens.*

Devatkal *et al.* (2005) standardized a PCR base method for the rapid detection of spoilage of buffalo liver. Template was prepared from buffalo liver stored at 4 ± 1°C for four days and PCR was repeated on days zero, two and four. The PCR amplifications were obtained with dilutions upto 10^{-4} and upto10^{-6} dilutions on day four. It was also noted that the intensity of bands decreased with increased dilutions and decreased number of bacteria.

For the enumeration of lactic acid bacteria in wine Neeley *et al.* (2005) developed a real time or quantitative PCR (QPCR). The QPCR consistently gave larger estimations of LAB populations than the plating results which was most likely due to the fact that QPCR amplified from all cells in wine both live and dead.

Rantsiou *et al.* (2008) described the development of a quantitative PCR (QPCR) technique to detect, quantify and determine the vitality of *L. monocytogenes* in foods. The method was based on the amplification of the intergenic spacer region between the 16S and 23S ribosomal RNA (rRNA) genes. A panel of more than 100 strains of Listeria spp. and non-Listeria was used in order to verify the specificity of the primers and Taqman probe and amplification signals were obtained only when *L. monocytogenes* DNA and RNA were loaded in the QPCR mix. Standard curves were constructed in several food matrices (milk, meat, soft cheese, fermented sausage, cured ham and ready-to-eat salad). The quantification limit was of $10^3 - 10^4$ cfu/g or ml, while for the determination of vitality it was 10^4–10^5 cfu/g or ml. After an overnight enrichment in BHI at 37°C also 10 cfu/g or ml could be detected in all the matrices used in this study. When the protocol was applied to food samples collected from the market or from small food processing plants, on a total number of 66 samples, four fresh cheeses from raw milk gave positive results prior to the overnight incubation, while nine samples, of which only one represented by fresh meat and the others by cheeses from raw milk, were positive after the enrichment. Out of the four

positive samples, only one could be quantified and it was determined to contain 4×10^3 cfu/g.

2.2 Pseudomonas species

Bacteria in the family Pseudomonadaceae are among the most important spoilage bacteria originating in refrigerated raw milk. Currently, the predominant microorganisms limiting the shelf life of processed fluid milk at 4°C are Pseudomonas species (spp). They are considered psychrotrophs, growing well at common refrigeration temperatures. In addition to the ability of Pseudomonas spp to grow to high numbers during refrigerated storage, many of these strains also produce heat-stable extracellular lipases, proteases, and lecithinases which can further contribute to milk spoilage. The heat stable lipases and proteases are responsible for milk defects such as bitterness, rancidity, fruity flavor, casein breakdown, and ropiness due to production of slime and coagulation of proteins. Many of these enzymes remain active, even following thermal processing steps that can destroy the organisms that produce these enzymes. Degradation of milk components through various enzymatic activities can reduce the shelf life of processed milk. Water and soil are the primary sources of Pseudomonas spp. Hose nozzles and milking equipment can become colonized by pseudomonads. The hydrolytic products of milk fats and proteins decrease the organoleptic quality of fluid milk products.

2.2.1 Conventional culture method

Grover and Srinivasan (1988) conducted a study for the isolation and characterization of *P. aeruginosa* from milk and milk products because of its public health significance. *P. aeroginosa* is responsible for many types of infections, food poisoning outbreaks and is wide spread in inadequately treated water supplies. The presence of *P. aeroginosa* was detected in 90.48 per cent of the samples tested.

Griffiths and Philips (1988), in their study on the relation between bacterial growth and storage temperature of pasteurized milk found that the shelf life of pasteurized milk was mainly determined by the level of contamination by gram negative psychotropic bacteria. At temperatures of 2°C, the greatest number of bacteria isolated from pasteurized milk belonged to genus Pseudomonas and its number decreased in proportion with increased temperature until they accounted only 10 per cent of the organisms that contributed to spoilage at 21°C.

Ternstrom *et al.* (1993) classified the spoilage flora of raw and pasteurized bovine milk. It was concluded that raw milk were exclusively spoilt by gram negative bacteria the majority of which were *P. fluorescens biovar I, P. fragi, P. lundensis and P. fluorescens biovar III.* Pasteurized milk was spoiled by the same gram negative organisms in 65 per cent (5°C) and 50 per cent (7°C) of the cases.

Sutherland *et al.* (1993) examined the factors that influenced the bacterial psychrotroph counts. Milk samples from five different dairies were examined over a period of 11 months and it was found that majority of the bacterial flora isolated were gram negative rods. Of the 76 gram negative rods 50 were Pseudomonas species 10 were Acenetobacter spp and six Flavobacteria spp.

Eneroth *et al.* (1998) reported that raw milk reached the critical bacterial loads of 10^6-10^7cfu/ml after approximately four days of storage at 7°C. They isolated gram negative psychotropic bacteria from all the samples and Pseudomonas were the most dominant spoilage microflora in all the incubated milk samples.

The study conducted by Jayarao and Wang (1999), revealed that *P. fluorescens* was the most predominant species isolated from bulk tank milk. Of the 116 isolates, 98 were Pseudomonas spp and remaining 18 were not identified. The study showed the importance of examining bulk milk tank for coliforms and non coliforms. The

enumeration of Pseudomonas spp was significant as they were closely associated with bacterial counts and milk quality.

Weidmann *et al.* (2000) isolated Pseudomonas spp from raw and processed milk. The predominant pseudomonas species were *P. putida, P. fluorescence* and *P. fragi.*

Dogan and Boor (2003) assessed the genetic diversity and spoilage potentials of Pseudomonas spp isolated from raw and pasteurized milk in four different dairy plants. They found that majority of the isolates were *P. fluorescens* and *P. putida* and that there existed high diversity among Pseudomonas species in the dairy plant and the strong association between Pseudomonas species ribotype and spoilage capacity.

Aaku *et al.* (2004) assessed the microbial quality of raw milk from two processing plants in Gaborone, Botswana and found that the organisms such as Pseudomonas which are normally eliminated by efficient pasteurization were still present in the commercially pasteurized milk samples. This suggested that the commercial pasteurization was rather inefficient or post pasteurization contamination has occurred.

Nanu *et al.* (2007) assessed the microbial quality of 240 raw milk samples obtained from the point of production (farmer's level) from Palakkad district in Kerala. The samples were also subjected to isolation of pathogenic and spoilage causing bacteria. Out of 240 samples, 26 (10.80 per cent) revealed the presence of *P. aeruginosa.*

2.2.2 PCR and related methods

Gutierrez *et al.* (1997) developed a quantitative PCR-ELISA for the rapid enumeration of bacteria in refrigerated raw milk. Primers were designed for the

amplification of a 147bp DNA fragment from a wide range of bacteria mainly of Pseudomonas species.

Spilker *et al.* (2004) used lysis buffer containing Sodium dodecyl sulfate and NaOH for the extraction of DNA from sputum isolates of patients with cystic fibrosis to detect P. aeruginosa which is an opportunistic pathogen.

Devatkal *et al.* (2005) standardized for rapid detection of spoilage of buffalo liver using a set of forward and reverse primers coding for conserved region of 23S rRNA gene present in *P. fragi.* The PCR was performed with DNA extracted from dilutions of fresh liver homogenate and after four days of storage at 4 ± 1°C.

2.3 *Escherichia coli*

Escherichia coli is present everywhere in the environment. It is often used by researchers as a basic research tool because it grows quickly and its behavior is similar to other disease-causing bacteria.. There are hundreds of strains of *E. coli.* Most *E. coli* strains are harmless and live in the intestines of healthy humans and animals. They are classified and numbered by the antigens that they produce. Some of them have acquired genes, either through mutation or from other organisms, that allow them to produce chemicals that are harmful to people. If harmful strains, like 0157:H7, ingested they cause disease. Toxins, or poisons the bacteria secrete, cause swelling of the intestinal wall, which is what causes severe gastrointestinal distress.

Escherichia coli have been used as a microbiological indicator for faecal contamination in food and water (Bej *et al.*, 1990).

Several methods are used for the detection and enumeration of *E. coli* cells in water and foods, like the Bacterial Analytical Manual gas production method, the LST-MUG (4-methylumbelliferyl-beta-D-glucuronide) assay method or most

probable number method. However those *E. coli* isolates which are negative for lactose and beta glucuronidase are not detectable by these methods (Hsu *et al.*, 1991).

The pathogenic *E. coli* comprise a very small percentage of the total *E. coli* present in raw milk. However raw milk and dairy products such as pasteurized milk and yoghurt contaminated with *E. coli*, have been the main cause of several outbreaks of milk borne diseases since the 1980s and thus remain a serious health risk (Padhye and Doyle, 1991).

Feng (1995) stated that bovine products were most often implicated in food borne infections with *E. coli* serotype O157:H7 and that raw milk was the reason for several outbreaks in the US and Canada.

2.3.1 Conventional culture method

Jayarao and Henning (2001) examined bulk tank milk from 131 dairy herds in eastern South Dakota and western Minnesota for the presence of for foodborne pathogens. *Campylobacter jejuni*, shiga-toxin producing *E. coli*, *L. monocytogenes*, Salmonella spp., and *Yersinia enterocolitica* were detected in 9.2, 3.8, 4.6, 6.1, and 6.1per cent of bulk tank milk samples, respectively. Thirty-five of 131(26.7 per cent) bulk tank milk samples contained one or more species of pathogenic bacteria.

Hundred raw milk and sixty milk product samples namely gulabjamun, mawa and dahi were randomly collected from different localities of Tandojam for the isolation of *E. coli*. All the samples were inoculated on different bacteriological media and a number of biochemical tests were performed for the confirmation of the isolate. The results revealed that out of the 100 samples, 57 per cent showed growth of *E. coli*. The highest numbers of milk samples contaminated with *E. coli* were recorded in milk samples obtained from vending shops and houses (Soomro *et al.*, 2002).

Lues *et al.* (2003) collected and analysed milk samples from 60 randomly selected households in the Botshabelo township for the presence of microorganisms. In a marked number of samples the total mesophilic, coliform and *E. coli* counts exceeded the national standard (5000 cfu/ml for total mesophilic counts; 10 cfu/ml for coliforms and absence of *E. coli* in 1ml for both raw and pasteurized milk intended for consumption). Both the total mesophilic and coliform counts exceeded the national standard in more than 80 per cent of the samples and reached counts of up to 10^{10} and 10^{9} cfu/ml, respectively. The *E. coli* counts were between zero and 10^{1} cfu/ml in 76.6 per cent of the samples whilst counts of up to 10^{5} were found in some instances. Although specific national guidelines in terms of aerobic and anaerobic spore-forming organisms, members of the Enterobacteriaceae family, yeasts and moulds have not yet been set, the counts of these microbiota were also very high. The data suggested that the general food hygiene related knowledge and infrastructure of the community was lacking, affirming the relationship between socio-economic status and household hygiene. The elevated microbial counts resulting from ignorance towards proper handling and housekeeping practices, pointed to milk as a sure medium of food-related infection in the area.

Mamani *et al.* (2003) collected samples of ultra heated whole milk and studied the growth and survival *E. coli* O157: H7 in milk stored at 4°C. *E. coli* counts were similar between the inoculum time and 4 days of storage at 4°C. Final counts after 68 days of storage at 4°C were about 1 -1.9 $\log_{10}$ cfu/ml in all samples.

Oksuz *et al.* (2004) examined 100 raw milk samples from different bovines and 50 white pickled cheeses manufactured from raw milk for the presence of *E. coli* O157. Furthermore, some physical and chemical properties were investigated. *E. coli* O157 was determined in one per cent of the total raw milk samples and in four per cent of the cheese samples.

Prejit (2005) isolated four strains of *E. coli* from 20 pooled raw milk samples obtained from the live stock farm of the Kerala Agricultural University.

Jaibi (2006) studied the microbial quality of a total of 144 raw milk samples, consisting of individual and pooled milk samples collected from three societies *viz.* S_1, S_2 and S_3 in Kerala. *E. coli* was isolated from 42 (38.89 per cent) individual milk samples and 17 (47.22 per cent) pooled milk samples.

Nanu *et al.* (2007) assessed the microbial quality of 240 raw milk samples obtained from the point of production (farmer's level) from Palakkad district in Kerala. The samples were also subjected to isolation of pathogenic and spoilage causing bacteria. Out of 240 samples, 76 (31.67 per cent) revealed the presence of *E. coli.*

Lekha (2006) identified a total of 45 isolates as *E. coli* out of the 108 raw milk samples collected from three societies in and around Mannuthy.

Asha (2007) isolated *Escherichia coli* from 57 per cent of the pooled raw milk samples from two of the dairy plants in Kerala.

Gini (2007) identified a total of 39 isolates as *E. coli* out of 108 raw milk samples analyzed from Thrissur.

Bhat *et al.* (2007) reported four county residents with laboratory-confirmed *E. coli* O157:H7 infection. All four residents reported having consumed raw milk obtained from a farm in neighboring Cowlitz County, Washington.

2.3.2 PCR and Related Methods

Allmann *et al.* (1995) developed a PCR to detect the presence of pathogenic microorganisms of dairy products. Here the total bacterial population of food sample

was isolated by centrifugation and analyzed by PCR specific for the pathogenic species. The study was conducted to detect the presence of *L. monocytogenes, E. coli,* enterotoxigenic *E. coli, Campylobacter jejuni and C. coli.* The approach was identical for any bacterial species to be detected except for different amplification primers.

Deng *et al.* (1996) used a combination of enrichment culturing, high temperature, denaturation and PCR for the detection of *E. coli* in food like ground beef chicken, pork and milk. The enrichment increased the viable cells, dilutes dead cells, high temperature inactivates some inhibitory substances which obviates the need for DNA extraction and purification.

Desmarchelier *et al.* (1998) detected *E. coli* O157 directly from milk by PCR based on the *rfb*E-O antigen synthesis genes. The PCR detected less than one cfu of *E. coli* O157 per ml of milk following enrichment.

McKillip *et al.* (2000) made a comparison of solvent extraction method and concentration method for the detection of *E. coli* O157: H7 from artificially contaminated dairy products using PCR. The total DNA extraction was higher for the solvent extraction method, whereas purity was higher for the concentration method. PCR detection limit varied from 10^1 to 10^4 cfu per ml with the specific dairy product. The mean A_{260}/A_{280} ratio for the solvent extraction method was 1.33 ± 0.14, while that for the concentration method was 1.83 ± 0.54.

Polymerase chain reaction has led to rapid and sensitive detection of *E. coli* from various clinical samples and various food types (Hsu and Tsen, 2001).

Aslam *et al.* (2003) used a rapid boil method to isolate DNA from bacterial cells in milk for subsequent use in PCR. The study was aimed at to detect shiga toxin producing *E. coli, L. monocytogenes* and Salmonella in milk.

only O157:H7 serotype showed *eae* virulence genes. The strain O157:H7 isolated possessed intimin type g1 and belonged to phage type 31.

Padhye and Doyle (1991) developed a sensitive, specific procedure for detecting *E. coli* 0157:H7 in food in less than 20 h. The procedure involved enrichment of 25 g of food in 225 ml of a selective enrichment medium for 16 to 18 h at 37°C with agitation (150 rpm). The enrichment culture was applied to a sandwich enzyme-linked immunosorbent assay (ELISA) with a polyclonal antibody specific for *E. coli* O157 antigen as the capture antibody and a monoclonal antibody specific for enterohemorrhagic *E. coli* of serotypes O157:H7 and O26:H11 as the detection antibody. The ELISA was completed within 3h. The sensitivity of the procedure, determined by using *E. coli* O157:H7 inoculated ground beef and dairy products, including different varieties of cheese, was 0.2 to 0.9 cell per g of food. A survey of retail fresh ground beef and farm raw milk samples with this procedure revealed that 3(2.8 per cent) of 107 ground beef samples and 11 (10 per cent) of 115 raw milk samples were positive for *E. coli* O157:H7. Most-probable-number determinations revealed *E. coli* O157:H7 populations of 0.4 to 1.5 cells per g in the three ground beef samples. In addition to being highly specific, sensitive, and rapid, this procedure was easy to perform and was amenable to use by laboratories performing routine microbiological testing.

2.4 *Staphylococcus aureus*

This bacterium is found on humans (skin, infected cuts, pimples, noses and throats) and has been associated with a wide range of foods including meat and meat products, poultry and egg products, salads (such as egg, tuna, potato and macaroni), cream-filled bakery products and pies, sandwich fillings, and milk and dairy products. In general, staphylococcal poisoning often occurs when a food has been handled a great deal (such as the chopping and handling involved in making a salad or sandwich) and is then left at temperatures above refrigeration which allow the

bacteria to multiply and produce toxins. Severe nausea, abdominal cramps, vomiting and diarrhoea occur one to six hours after eating. Recovery occurs within two to three days or longer if severe dehydration occurs.

Bacteria are the causative agents of two thirds of food-borne disease outbreaks. Among the predominant bacteria involved in these diseases, *S. aureus* was the leading cause of gastroenteritis resulting from the consumption of contaminated food (Le Loir *et al*., 2003).

2.4.1 Conventional culture method

Yadava *et al.* (1985) reported that *S. aureus* constituted a significant proportion (19.04 per cent) of pathogenic flora of market milk which posed a high risk to the consumers. The percentage of isolation of *S. aureus* was higher from source with low bacterial count which indicated that *S. aureus* is outgrown by other competitive organisms.

Jolly *et al.* (2000) studied 60 raw market milk samples obtained from three sources *viz.* A, B and C located in and around Mannuthy. From each source, 10 individual and 10 pooled milk samples were collected. The study revealed that *S. aureus* was present in 50 per cent of pooled and 36.67 per cent of individual milk samples.

Lekha (2006) confirmed a total of 33 isolates from milk samples collected from three societies in and around Mannuthy, as *S. aureus* by cultural morphological and biochemical reactions.

Asha (2007) isolated *Staphylococcus aureus* from 57 per cent pooled raw milk samples from two dairy plants in Thrissur, which indicated the poor hygienic practices and poor health conditions of animals.

Gini (2007) identified a total of 44 isolates out of 108 raw milk samples from Thrissur as coagulase positive *S. aureus*.

Nanu *et al.* (2007) assessed the microbial quality of 240 raw milk samples obtained from the point of production (farmer's level) from Palakkad district in Kerala. The samples were also subjected to isolation of pathogenic and spoilage causing bacteria. Out of 240 samples, 84 (35 per cent) revealed the presence of *S. aureus.*

2.4.2 PCR and related methods

Phuektes *et al.* (2001) developed a multiplex PCR for the simultaneous detection of mastitis pathogens *S. aureus, Streptococcus dygalatiae, Streptococcus agalatiae, and Streptococcus uberis.* It was compared with conventional culture and results suggested multiplex PCR could be used as an alternative method in routine diagnosis as it was rapid sensitive and specific for the simultaneous detection of the four pathogens.

Riffon *et al.* (2001) made a comparative study between two PCR assays to develop a rapid sensitive and specific molecular method to identify pathogens involved in intramammary infections. The two PCR assays were performed on *S. aureus* samples prepared one with and other without pre enzymatic lysis step in milk and tryptic soy broth (TSB). Milk was compared with TSB to compare the inhibitory effect of milk. Sensitivity was higher for the pre PCR enzymatic lysis step using Qiagen kit than in the absence of pre PCR enzymatic lysis step. The level of sensitivity was higher in TSB than in milk.

Ramesh *et al.* (2002) evaluated the chemical extraction method of bacterial DNA which was a combination of organic solvents, detergents and alkali. The method provided a direct detection limit of 10 cfu per ml milk spiked with *S. aureus* and *Y. enterocolitica*. The use of SDS and NaOH helped the efficient lysis of robust

gram positive bacterial cells which would otherwise require the use of enzyme lysozyme and proteinase K for the elaboration of nuclease from the bacterial cells.

Nakano *et al.* (2004) designed primers targeting 16SrRNA gene for the detection of bacteria belonging to Bacillus and Staphylococcal genera. Specificity was determined with 81 different bacterial strains and eukaryotes. Bacterial DNA was extracted with achromopeptidase and chelex 100 resin which followed a 5 h enrichment culture.

Enterotoxin genes (*sea-see*) in *S. aureus* isolates recovered from milk of clinically healthy sheep and cows in the north of Palestine were determined using a polymerase chain reaction (PCR). Thirty-seven (37 per cent) out of 100 *S. aureus* isolates were toxin gene positive. Four strains (10.8 per cent) were *sea*-positive, 20 (54.1 per cent) were *seb*-positive, 4 (10.8 per cent) were *sec*-positive, 6 (16.2 per cent) were *sed*-positive and 3 (8.1 per cent) were *see*-positive. None of these enterotoxigenic isolates carried more than one toxin gene. The study indicated that the presence of enterotoxigenic *S. aureus* in raw milk contributed to the sources of staphylococcal food poisoning in Palestine (Adwan *et al.*,2005).

Nakayama *et al.* (2006) developed a direct detection system for eight Staphylococcal enterotoxin encoding genes. The combination of DNA preparation method and real time PCR was completed within four hours. The method included alkaline treatment of sample for cell lysis, petroleum ether extraction to eliminate fats and proteins, followed by the application of commercial kit for DNA purification and concentration.

Peles *et al.* (2007) characterized *S. aureus* isolates recovered from mammary quarter milk of mastitic cows and from bulk tank milk produced on Hungarian dairy farms of different sizes. Macrorestriction analysis of chromosomal DNA from *S. aureus* isolates was performed using the restriction enzyme *SmaI* followed by pulsed-

field gel electrophoresis (PFGE). The prevalence rates of nine *S. aureus* enterotoxin genes (*sea, seb, sec, sed, see, seg, seh, sei,* and *sej*) and of the toxic shock syndrome toxin 1 gene (*tst*) were determined by multiplex PCR. The bulk tank milks of 14 out of 20 farms were contaminated with *S. aureus* at levels of up to 6.0×10^3 cfu/ml. The prevalence rates of penicillin resistance were 88.9 per cent and 20.0 per cent among the *S. aureus* recovered from mastitic quarter milk and bulk tank milk, respectively. After phenotypic characterization, a total of 59 *S. aureus* isolates were selected for genotyping. The PFGE analysis revealed 22 distinct pulsotypes, including 14 main types and 8 subtypes, at a similarity level of 86 per cent. Only one or two main types were observed on each of the farms tested, indicating a lack of genetic diversity among *S. aureus* isolates within farms, and there were only two pulsotypes which occurred on more than one farm. The PFGE patterns showed genetic relatedness between the *S. aureus* strains recovered from quarter milk and bulk milk on two large farms, implying that on farms having a high number of mastitic cows, *S. aureus* from infected udders may contaminate bulk milk and, subsequently, raw milk products. Sixteen (27.1 per cent) of the *S. aureus* isolates tested by multiplex PCR were found to be positive for enterotoxin genes, with 15 of them carrying just one gene and one strain carrying two genes (seg and sei). The most commonly detected toxin genes were *seb, sea,* and *sec*, whereas none of our isolates possessed the *see, seh, sej,* or *tst* genes. On 75 per cent of the dairy farms surveyed, no enterotoxigenic staphylococci were recovered from either mastitic quarter milk or bulk tank milk.

Yang *et al.* (2007) amplified the thermostable nuclease gene (*nuc*) for the detection of *Staphylococcus aureus.* A DNA fragment of 279 base pair (bp) was amplified by PCR. Only the DNA of *S. aureus* could be amplified using the primers.

Rall *et al.* (2008) analyzed the frequency of genes encoding the staphylococcal enterotoxins (SEs) SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ in *S. aureus* strains isolated from raw or pasteurized bovine milk. *S. aureus* was found in 38 (70.4

per cent) out of 54 raw milk samples at concentrations of up to $8.9x10^5$ cfu/ml. This microorganism was present in eight samples of pasteurized milk before the expiraty date and in 11 samples analyzed on the expiry date. Of the 57 strains studied, 68.4 per cent were positive for one or more genes encoding the enterotoxins, and 12 different genotypes were identified. The gene coding for enterotoxin A, *sea*, was the most frequent (16 strains, 41 per cent), followed by *sec*, (eight strains, 20.5 per cent), *sed* (five strains, 12.8 per cent), *seb* (three strains, 7.7 per cent) and *see* (two strains, 5.1 per cent). Among the genes encoding the other enterotoxins, *seg* was the most frequently observed, (11 strains, 28.2 per cent), followed by *sei* (10 strains) and *seh* and *sej* (three strains each). With the recent identification of new SEs, the perceived frequency of enterotoxigenic strains has increased, suggesting that the pathogenic potential of staphylococci may be higher than previously thought; however, further studies are required to assess the expression of these new SEs by *S. aureus*, and their impact in food-borne disease.

2.5 DNA EXTRACTION

Wegmuller *et al.* (1993) isolated the bacterial cells from dairy products using digestion buffer containing Tris EDTA, SDS and pronase, followed by centrifugation. The cells were then lysed by lysozyme and proteinase K digestion for preparation of lysates for PCR.

Muramatsu *et al.* (1996) stated that the combination of direct Immunomagnetic separation (IMS) and PCR was suitable for detecting Coxiella. The combination took only 5 h to visualize the amplified products, because direct IMS can easily and rapidly yield the sample for PCR from milk. For detection by PCR, the method of recovery is no less important than the selection of the pair of primers. The combination of direct IMS and PCR is thought to be applicable to detect Coxiella from raw cow milk.

Gutierrez *et al.* (1997) used a commercially available milk clearing solution which facilitated the recovery of bacterial cells without the components which inhibit the PCR reaction. The recovered cells were then lysed in lysis buffer, the DNA precipitated with isopropanol and transferred into TE buffer.

Lindqvist (1997) described a sample treatment method based on buoyant density centrifugation which separates bacteria from food, concentrates bacteria and removes PCR inhibitors. The method involved a one minute centrifugation of food homogenate layered over a gradient medium (PercoIl@ or BacXtractorTM) in Eppendorf tubes, followed by a single wash step. Centrifugation for one minute at 16500g was found to be optimum to separate the bacteria from food to the bottom of centrifuge tube. At low g forces bacteria adhered along the sides of the eppendorf tube. The small scale of this treatment makes it possible to process many samples in a short time. To evaluate the method beef and minced beef samples, spiked with strains of *E. coli* O157:H7, were treated and then analysed by PCR aimed at verocytotoxin- *(VT1* and *VT2)* and *ene*-genes. The detection limits in 1:10 (w/v) beef and minced beef homogenates were 125-250 cfu/ml (1250-2500 cfu/g) and 1000 cfu/ml (1 x I0 cfu/g) respectively. The enrichment of spiked samples in buffered peptone water at 37°C for six hours before buoyant density centrifugation and PCR, allowed 0.5 cfu/g beef and five cfu/g minced beef to be detected. This combination of enrichment and buoyant density centrifugation was also used for analysis of 43 beef samples from a consignment in which E. *coli* O157:H7 had been detected, and detected *VT*-genes in all 43 samples. *E. coli* O157:H7 was also separated and detected in spiked samples of milk, lettuce, shrimps, and blue cheese at arbitrary concentrations of 3000 cfu/ml. The present sample preparation method has the potential to be applicable to many other combinations of bacteria and food, and in connection with other detection methods than PCR as well.

Wang *et al.* (1997) developed a universal protocol for the PCR detection of 13 species of food borne pathogens. Samples were prepared with overnight incubation at 37°C before DNA extraction and PCR.

According to Winters *et al.* (1998) traditional microbiological isolation and identification methods took several days to complete, so there is need for a more rapid, sensitive and specific methods for identification of contaminated food products. So study was conducted in which PCR was used to detect food borne pathogens. Food samples artificially contaminated with *C. jejuni* were used for the study. Each food was then used for PCR assay without prior enrichment of the contaminating organism. Although the food storage conditions were not associated with the growth of *C. jejuni* the length of time for which the cell survived at ambient and refrigeration temperatures was of significance because the infectious dose may be as low as 500-800 cells.

Romero and Lopez-Goni (1999) modified the DNA extraction methods, as inefficient DNA extraction could account for PCR negative results. They found that a positive PCR was obtained when DNA extraction was performed with SDS in NET buffer. They reported that the use of NET buffer with high concentrations of EDTA, Tris, SDS and proteinase K were necessary for efficient lysis of cell envelope of Brucella species, compared with that of highly sensitive cell envelope of other gram negative bacteria. This DNA purification method can be applied to PCR detection of other bacterial pathogens in milk.

Hsu and Tsen (2001) extracted DNA after sedimentation of the target cells by centrifugation, which was then incubated with proteinase K at 65°C for 30 minutes and then directly used in PCR.

Phuektes *et al.* (2001) used phenol chloroform extraction of DNA. The method took less than 24h to identify bacteria upto species level.

Cremonesi *et al.* (2006) accomplished the efficient lysis of cells by increasing the concentration of guanidine thiocyanate and lysis buffer, which increased the disruption of bacterial cells resulting in stronger and more reproducible amplification, avoiding the combination of enzymes and incubation conditions. The sample processing time took approximately 90 minutes and whole procedure including DNA extraction, PCR amplification and gel band visualization was completed in six hours.

Furet *et al.* (2004) used a commercial kit according to the manufacturer's instructions for direct DNA extraction of gram positive bacteria from raw milk.

Amagliani *et al.* (2004) used two magnetic based methods for rapid and direct detection of *L. monocytogenes* in milk, both of which showed elevated level of specificity and sensitivity with detection level upto 10 cfu per ml. The time required for magnetic extraction of bacterial DNA, PCR and gel electrophosis was approximately one working day.

Gillespie and Oliver (2005) evaluated several methods for the isolation of bacterial DNA directly from milk. It was found that although the methods that used proprietary reagents such as InstaGene Matrix (Biorad) and Prepman Ultra reagent (Applied Biosystems) were faster and convenient, the results were not consistent. It was found that the method described by Allmann *et al.* (1995), was the most consistent and reproducible one. The method involved overnight enrichment with tryptic soy broth at 37°C, digestion with pronase to remove fat, centrifugation to pellet the cells and lysis by lysozyme and proteinase K.

Di- Pinto *et al.* (2007) compared the DNA extraction efficiency of two procedures for food analysis namely Wizard Magnetic Purification for food (Promega, Italy) and DNeasy Tissue Kit (Qiagen, Germany). The former had a high efficiency for vegetable matrices, whereas the latter was feasible for routine analysis on dairy and meat products. However, both gave satisfactory results for most food

samples with lower amount of potential contaminants compared to other techniques, like the hexadecyltrimethyl ammonium bromide (CTAB) based extractions which may have contaminants of CTAB, which reduced the efficiency of PCR.

Yang *et al.* (2007) carried out solvent extraction procedure for the extraction of DNA of *S. aureus* directly from whole milk, skim milk, and cheese. It allowed the detection of *S. aureus* in dairy products in less than six hours which was less than the time taken by the general PCR assay using the enrichment method.

Yang *et al.* (2007) developed a method combining nanoparticle-based immunomagnetic separation (IMS) with real-time PCR for a rapid and quantitative detection of *L. monocytogenes*. Carboxyl modified magnetic nanoparticles were covalently bound with rabbit anti-*L. monocytogenes* via the amine groups. Several factors, such as the amount of immunomagnetic nanoparticles (IMNPs), reaction and collection times, and washing step, were optimized, and the nanoparticle-based IMS in combination with real-time PCR was further evaluated for detecting *L. monocytogenes* from artificially contaminated milk. The cell numbers calculated from the means of threshold cycles (CT) of PCR amplification curves were compared to those from plate counts in order to determine the correspondence degree of quantitative data. The capture efficiency (CE) by plating from IMNP-based IMS was 1.4 to 26 times higher than those of Dynabeads-based IMS depending on the initial cell concentrations inoculated into milk samples. When combined with real-time PCR, *L. monocytogenes* DNA was detected in milk samples with *L. monocytogenes* more than 10^2 cfu/0.5 ml. In the range of 10^3 to 10^7 *L. monocytogenes* cfu/0.5 ml, cell numbers calculated from CT values were 1.5 to 7 times higher than those derived from plate counts. The results demonstrated that both the use of nanoparticles and the choice of anti-*L. monocytogenes* in IMNP based IMS in combination with real-time PCR has improved the sensitivity of *L. monocytogenes* detection from both nutrient broth and milk samples.

2.6 POLYMERASE CHAIN REACTION

The concept of PCR was first described by Panet and Khorana, (1974) and owes its name to Dr Karry Mullis and colleagues who developed the process over the course of four months in 1983 at the Cetus Corporation. In this in vitro laboratory procedure, the template DNA strands get separated on heating. The primers and annealing temperature at which the primers anneal to the template are critical for the specificity of the reaction. The extention temperature allows primer extension. In a 25 to 35 cycle reaction many million copies of the sequence is produced which can be detected by agarose gel electrophoresis by staining with ethidium bromide and photographing under ultraviolet light illumination.

Bej *et al.* (1990) to overcome the problems inherent in methods based on the culturing of viable cells for the detection of coliforms developed a non culture genetically based procedure for the environmental detection of coliforms based on the recovery of DNA, amplification of target nucleotide sequences specially associated with coliform bacteria using the polymerase chain reaction and detection of the amplified DNA with gene probes.

According to Hill (1996), a positive PCR result is not conclusive that viable cells are present in the sample, as dead cells contain amplifiable DNA. But the presence of dead cells too is of significance as it indicates that the product was not prepared from ingredients of sufficient quality or viable cells were present at the time food was consumed which poses a health hazard.

Henegariu *et al.* (1997) compared the effect of varying concentrations of dNTP and magnesium concentration on multiplex PCR and found that increasing the dNTP concentrations inhibited PCR, whereas, increase in magnesium concentration often had positive effects.

2.6.1 PCR based assays

Fach and Popoff (1997) could detect *Clostridium perfringens* by conventional PCR at 10 cfu/gram in meat, milk and salad following enrichment.

Oberst *et al.* (1998) stated that gel electrophoresis did not show the sensitivity and specificity of PCR. 5' nuclease assay was used that allowed the 5'-3'exonuclease activity of *Thermus aquaticus* DNA polymerase to hydrolyse an internal TaqMan probe labeled with a fluorescent reporter dye and a quencher dye. Due to the proximity to the probe, the quencher dye suppressed the fluorescent emission of the reporter dye. During PCR, the probe anneals to the target amplicon and gets hydrolysed, which reduced the quenching effect. This resulted in increased emission of reporter fluorescence. Interpretation of fluorometric data was automatically read and interpreted to give a yes or no conclusion to the presence or absence of DNA within 15 minutes of completion of PCR.

Straub *et al.* (1999) detected *S. aureus* 5-15 cfu per gram in skim milk and cream by PCR following enrichment.

Lucore *et al.* (2000) developed a bacterial concentration method based on the selective adsorption of bacteria to metal hydroxides in solution followed by low speed cocentration to concentrate the cells (*L. monocytogenes* and *Salmonella enteritidis*) from the food matrix (artificially contaminated milk).

McKillip *et al.* (2000) concentrated bacteria using metal hydroxides and centrifugation followed by guanidine isothiocynate extraction for detection of *E. coli* O157: H7 from artificially contaminated dairy products.

Phuektes *et al.* (2001) found that multiplex PCR had a 10-100 fold lower sensitivity compared to simplex PCR when tested on DNA extracted from each of the target pathogens, namely *S. aureus, S. dygalatiae, S. agalatiae and S. uberis.*

Meiri-Bendek *et al.* (2002) developed a PCR based method for the detection of *S.agalactiae* in milk. Selective enrichment of *S. agalactiae* was done in milk samples followed by DNA extraction and PCR. The specificity of PCR was based on DNA sequences within the 16SrRNA genes of *S. agalactiae.*

Aslam *et al.* (2003) detected DNA fragment amplified by PCR from as low as 10 picogram of purified DNA. The detection limit for PCR was as low as 10^2 cfu of bacteria per ml of milk in which Salmonella was grown overnight. When bacteria were added to milk and DNA was extracted immediately by rapid boil method the detection limit of PCR was as low as 5 cfu of bacteria per ml of milk or Phosphate Buffered Saline (PBS). The decrease in sensitivity of PCR detection when bacteria were grown overnight in milk was attributed to the coating of bacteria with milk fat and consequent insufficient bacterial lysis.

Moon *et al.* (2004) examined the effects of food matrix including milk, raw chicken meat, ham and cheese for the sensitivity of the PCR, populations of *E. coli* O157: H7 and *L. monocytogenes* after enrichment at 37°C overnight were counted on cefixime tellurite-sorbitol MacConkey agar and Oxford Listeria selective agar with supplement (LSA) respectively. Upon PCR amplification, *E. coli* O157: H7 was detected at 10^4 to 10^0 initial cfu/ml before and after enrichment, respectively which were even higher for Listeria. The result indicated milk as a good media for cultivation of *E. coli* O157: H7 but not for *L. monocytogenes.*

Gupta *et al.* (2006) always obtained a positive PCR result with different aliquots containing 2.2 X 10^6 cfu per ml of milk. To determine sensitivity of ten fold serial dilutions of *B. melitensis* pure culture was prepared in one ml of raw milk. PCR

was found to be 90 percent sensitive and cent percent specific in comparison to 70 per cent and 80 per cent sensitive and specific respectively in serology.

Arora *et al.* (2006) compared ELISA, PCR and culture methods for detecting Aeromonas species in foods of animal origin. Direct application of PCR to food samples often resulted in absence of detectable amplification product which was overcome by an enrichment step. Finally, it was concluded that both ELISA as well as duplex PCR following 12 hour enrichment were reliable of the three methods compared.

2.6.2 Primer

Barry *et al.* (1991) examined the spacer sequences and found that there exists extensive sequence variation between microorganisms which should be species specific. Based on this, oligonucleotide PCR primer were synthesized that could amplify 16S/23S spacer sequences of a number of species of genus Clostridium to examine the efficacy of the method.

Jensen *et al.* (1993) developed a unified set of primers and polymerase chain reaction conditions to amplify spacer regions between the 16S and 23S genes in the prokaryotic RNA genetic loci. This set of primers and reaction conditions were applied for the amplification of 16S-23S ribosomal spacer regions for over 300 strains of bacteria belonging to eight genera and 28 species. The results showed that amplification of 16S-23S ribosomal spacer region is suitable for identification of bacteria at species level.

Meng *et al.* (1996) designed a pair of oligonucleotide primers which were used in polymerase chain reaction to amplify a 633 base pair (bp) fragment of a target gene encoding an outer membrane protein of *E. coli* O157:H7 and O55.

Forsman *et al.* (1997) found significant variation between the 16S-23S spacer sequences of the nine different species that cause bovine mastitis. This variation made it possible to construct specific primer pairs for these species.

Venkitanarayana *et al.* (1997) for the estimation of aerobically stored meat by quantitative polymerase chain reaction selected primers from a conserved 23S ribosomal DNA (rDNA) sequence of *P. aeroginosa* as it was shown to amplify the DNA of nine spoilage bacteria as a group.

According to Gutierrez *et al.* (1998) it would be appropriate to design primers for a conserved region of the DNA that would detect the majority of spoilage bacteria as a group for a given commodity because it was more economical and required less time than an assay for each individual species of spoilage bacteria. As the spoilage is mainly of economic significance there is no need to detect them individually in foods.

Marcos *et al.* (1999) used the *S. aureus ara* gene as a target for the amplification of 1153 bp DNA fragment by PCR. The products were analyzed by agarose gel electrophoresis. The detection limit was 20 cfu when cells were suspended in saline and $5x10^2$cfu per ml when *S. aureus* cells were suspended in sterilized whole milk. No PCR products were obtained when other Staphylococcal species or genera were analyzed with the same primers.

Phuektes *et al.* (2001) used oligonucleotide primers from the 16S to 23S RNA intergenic spacer regions for *S. aureus, S. dysgalatiae, S. agalatiae* and *S. uberis* in the multiplex PCR for their simultaneous detection.

Martinez *et al.* (2001) developed a method based on polymerase chain reaction using species specific and universal primers derived from 16S rRNA gene for the direct detection of *S. agalactiae* from cow's milk.

Daly *et al.* (2002) developed a PCR for the detection of *E. coli* in milk. Primers specific for conserved region in the *E. coli* alanine racemase gene were selected. Alanine racemase is produced by all strains of *E. coli* including O157 and is essential for cell wall synthesis by providing D-alanine to peptidoglycan.

Kuzma *et al.* (2003) used primers specific for *nuc* gene of *S. aureus* for PCR amplifications. These primers could not detect and amplify DNA from milk samples infected with bacteria other than *S. aureus* and the samples negative by culture.

Spilker *et al.* (2004) used 16S rDNA sequence data to design two PCR assays, one specific for genus Pseudomonas, while the other specific for *P. aeruginosa.*

2.6.3 PCR inhibitors

PCR inhibitors are substances that chelate or degrade a component in the PCR reaction mixture. They prevent the participation of the components in the reaction thus inhibiting the DNA synthesis.

Wilson (1997) stated that the inhibitors of amplification of nucleic acids by PCR act in three essential ways that is they interfere with the cell lysis necessary for extraction of DNA, they interfere by nucleic acid degradation or capture or they inhibit the polymerase activity for amplification of target DNA. The inhibitors in milk are components such as calcium, proteinase, fats and milk proteins which lock DNA or shield it from access by polymerase.

2.6.3.1 Removal of PCR inhibitors

Inhibition from food can be reduced by efficient sample preparation techniques like concentration of the target cells, dilution of the food matrix and removal of chelators.

According to Murphy *et al.* (2002) the genomic DNA isolated directly from milk would be severely degraded as the casein interferes with proteinase K digestion of enzymes that degrade somatic cell DNA. So initial treatment with EDTA was done to dissolve casein micelles prior to proteinase K digestion which improved the quality of genomic DNA.

Kuzma *et al.* (2003) decreased the number of false negative by decreasing the amount of template from 5 µl to 2 µl in a 50 µl reaction. Decreasing the concentrate of template was compared with dilution of samples which was a method for removal of amplification inhibitors.

Fode-vaughan *et al.* (2003) found that there was lower abundance of PCR product in 1:10 dilution containing 100 cells compared to 1:100 dilution containing 10 cells which was attributed to the probable PCR inhibitory substances in milk.

Wu and Kado (2004) diluted the *E. coli* O157:H7 inoculated milk in Luria-Bertani broth to decrease the concentration of potential PCR inhibitors. The DNA extraction was done following incubation at 37°C for four hours and subsequent filtration of *E. coli* cells using a filtration unit.

2.6.4 PCR facilitators

Certain substances have the ability to improve the amplification efficiency of DNA polymerases in the presence of inhibitors present in blood, food and faeces.

Al–Soud and Radstrom (2000) studied the effect of 16 PCR facilitators on the amplification capacities of rTth and Taq DNA polymerases in the presence of inhibitory samples like blood meat and faeces. Four facilitators (11.7 per cent Betaine, 0.4 per cent Bovine Serum Albumin, 0.01 per cent gp32, and 1X proteinase inhibitor mixture) which had the highest relieving effects were selected to study the effect of their combinations on the amplification capabilities of the DNA

polymerases. Among the 16 facilitators, bovine serum albumin reduced the inhibition of both the polymerases in the presence of all type of inhibitory samples. Only Betaine was found to enhance the fluorescence signal in the presence of all inhibitors. The combination of various facilitators had no synergistic or additive effects.

Daly *et al.* (2002) included betaine (one mol/litre) as a PCR additive in the reaction mixture to enhance amplification for the detection *E. coli* in milk.

Materials and Methods

3. MATERIALS AND METHODS

The study was conducted for a rapid assessment of the hygienic quality of milk using PCR. Assessment of bacterial load in milk by PCR using Pseudomonas, the predominant spoilage organism was compared with conventional culture method. DNA from varying concentrations of Pseudomonas in milk was isolated; PCR assayed and compared with the aerobic plate count by conventional culture to find out the bacterial load upto which Pseudomonas in milk could be detected. The band pattern was compared with the count obtained by the conventional aerobic plate count technique. In addition PCR was employed for rapid detection of specific pathogens *Escherichia coli and Staphylococcus aureus* directly from milk. For standardization of DNA extraction, sterile milk was first spiked with varying concentration of *E. coli and S. aureus* followed by PCR assay. The minimum detection level of pathogens in milk was determined. DNA extraction was carried out from organisms inoculated into PBS as well as milk to note the difference in milk as a food matrix and also for potential inhibitors in milk. The extracted DNA was directly used as template in PCR. The sensitivity of PCR for detection of pathogens from PBS and milk was also recorded. To determine the specificity, PCR was performed with species specific primers, from milk samples containing varied microflora. The optimized PCR protocol was then used for the analysis of 50 milk samples.

3.1. STANDARDIZATION OF DNA EXTRACTION

3.1.1 Materials

3.1.1.1 Phosphate buffered saline (HiMedia, Mumbai.)

To wash the pelleted cells after centrifugation.

3.1.1.2 Sodium chloride - molecular weight (MW) 58.44 (Sisco Research Laboratories, Mumbai.)

Dissolved 5.844 gram (g) of Sodium chloride in 100 ml triple distilled water to prepare a stock solution of 1M concentration (100ml).

3.1.1.3 Tris HCl - MW 157.6 (Sisco Research Laboratories, Mumbai.)

Tris HCl (7.88 g) was dissolved in around 98 ml triple distilled water and pH was adjusted to 7.4 with sodium hydroxide crystals to prepare a stock solution of 0.5 M Tris HCl (pH 7.4)

3.1.1.4 Ethylene Diamine Tetra Acetic acid (EDTA) – MW 372.2 (Sisco Research Laboratories, Mumbai.)

EDTA (3.722 g) was dissolved in about 95 ml triple distilled water and pH was adjusted to 7.4 with sodium hydroxide crystals to prepare a stock solution of 0.1 M EDTA (pH 7.4)

3.1.1.5 Sodium Dodecyl Sulfate (SDS 10%) **(Bangalore Genei)**

Sodium Dodecyl Sulfate is a detergent commonly used in DNA extraction. It causes the rupture of cell wall and nuclear membrane to release the contents. It also dentures the proteins.

3.1.1.6 Sodium Tris EDTA buffer (NTE Buffer) containing 0.5%SDS

1Molar (M) sodium chloride	10 ml
0.5M TrisHCl (pH 7.4)	4 ml
0.1M EDTA (pH 7.5)	1 ml
SDS 10%	5 ml

3.1.1.7 Proteinase K (Bangalore Genei)

Enzyme commonly employed for digestion of proteins. It is a highly active protease purified from mold *Tritirachium album.* The digestion with proteinase K is usually carried out in the presence of EDTA because EDTA inhibits the action of magnesium ion dependent nucleases which otherwise can digest the DNA.

3.1.1.8 Lysozyme (Bangalore Genei)

Enzyme used for digestion of gram positive cell wall.

3.1.1.9 Tris Suturated Phenol (Bangalore Genei)

Phenol used to extract the DNA from solution. In alkaline pH it extracts the DNA to aqueous phase which is collected for further purification. This will prevent the contamination of DNA with RNAs. In neutral or acidic pH phenol extracts RNA to aqueous phase. Hence the pH of phenol (more than 7.8) is very important.

3.1.1.10 Chloroform (Sisco Research Laboratories, Mumbai.)

It is used for denaturation of proteins

3.1.1.11 Isoamyalcohol (Sisco Research Laboratories, Mumbai.)

To reduce foaming and to maintain the stability of layers of deproteinised solution after centrifugation.

3.1.1.12 Sodium Acetate (Sisco Research Laboratories, Mumbai.)

Dissolved 24.609 g sodium acetate (MW 82.03) in approximately 60 ml of triple distilled water and made upto 100 ml with glacial acetic acid to prepare 3M sodium acetate (pH 4.8). Sodium Acetate provides the necessary environment for the precipitation of DNA.

3.1.1.13 Ethanol (100%)

It is used precipitate the DNA.

3.1.1.14 Ethanol (70%)

100%ethanol – 70ml

Triple distilled water – 30ml

Stored at 4°C until use. To wash the precipitated DNA.

3.1.1.15 Triple distilled water

Triple distilled water was prepared in our laboratory with triple distillation apparatus. It was used to dissolve the DNA precipitated to be used as template in PCR.

All chemicals used were of molecular grade or extrapure.

3.1.1.16 Bacerial strains

The control strains of *Pseudomonas aeroginosa*, *Escherichia coli*, and *Staphylococcus aureus*, were obtained from MTCC (Microbial Type Culture, Chandigarh). Bacterial cultures were grown in nutrient broth at 37° C before DNA extraction.

3.1.2 Method

3.1.2.1 Preparation of template DNA of Pseudomonas Aeruginosa from culture (Boiling lysis)

Pure culture of *P. aeruginosa* was grown in nutrient broth overnight. The culture was taken in separate eppendorf tube (1.5 ml) and centrifuged at 3000g for 10

minutes. The supernatant was discarded and the pellet obtained at the bottom of the tube was washed twice with chilled sterile PBS and finally the pellet was resuspended in 100µl of triple distilled water. The mixture was boiled for 10 minutes and then immediately chilled on ice for 30 minutes. The samples were thawed and centrifuged at 3000g for five minutes and the supernatant was stored at -20°C for further use as template for PCR (Flow Chart 1.).

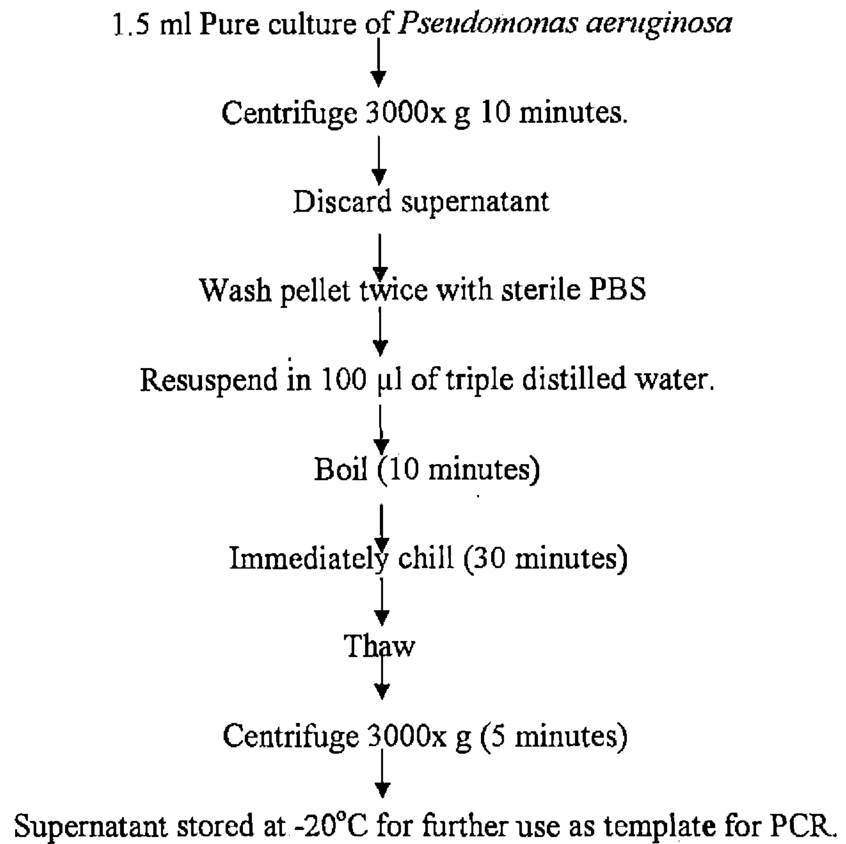
3.1.2.2 Preparation of template DNA of Escherichia coli from culture (Boiling lysis)

The same procedure as above with pure culture of *E. coli.*

3.1.2.3 Preparation of template DNA from pure culture of gram negative bacteria (Pseudomonas aeruginosa and Escherichia coli) by phenol chloroform extraction

Phenol chloroform method of DNA isolation (Phuektes *et al*, 2001) was used for DNA extraction from pure culture of *P. aeruginosa*. To 300 µl of pure culture of *P. aeruginosa* taken in a 1.5 ml microcentrifuge tube, 300µl NTE buffer containing 0.5 %SDS and 100µg of proteinase K per ml was added. The solution was then incubated at 37°C for four hours. An equal volume of phenol was added, and the solution was gently mixed for three minutes. The solution was centrifuged for three minutes at 10000x g, and the upper phase was collected without disturbing the interphase. This process was repeated once. Then an equal volume of chloroform isoamylalcohol (24:1) was added and the solution was gently mixed for three minutes. The solution was centrifuged for three minutes at 10000x g. The upper phase was collected and 60µl of 3M sodium acetate and 1.2ml of cold 100% ethanol was added. The solution was mixed and held at -40^{0}C for 30 minutes to precipitate the DNA. The DNA was recovered by centrifugation at 10000x g for 15 minutes at 4^{0}C. The supernatant was discarded and the pelleted DNA was washed with 70% ethanol and centrifuged at 10,000x g for five minutes at room temperature. The DNA

Flow chart 1. Preparation of template DNA of *Pseudomonas aeruginosa/ Escherichia coli* from culture (Boiling lysis)



pellet was then air dried and 50µl of triple distilled water was added to dissolve the DNA (Flow Chart 2.).

The same procedure was followed for *E. coli* too.

3.1.2.4 Preparation of template DNA from pure culture of gram positive Staphylococcus aureus (phenol chloroform extraction)

The above procedure with the addition of lysozyme along with NTE buffer at the rate of 1mg/ml was done.

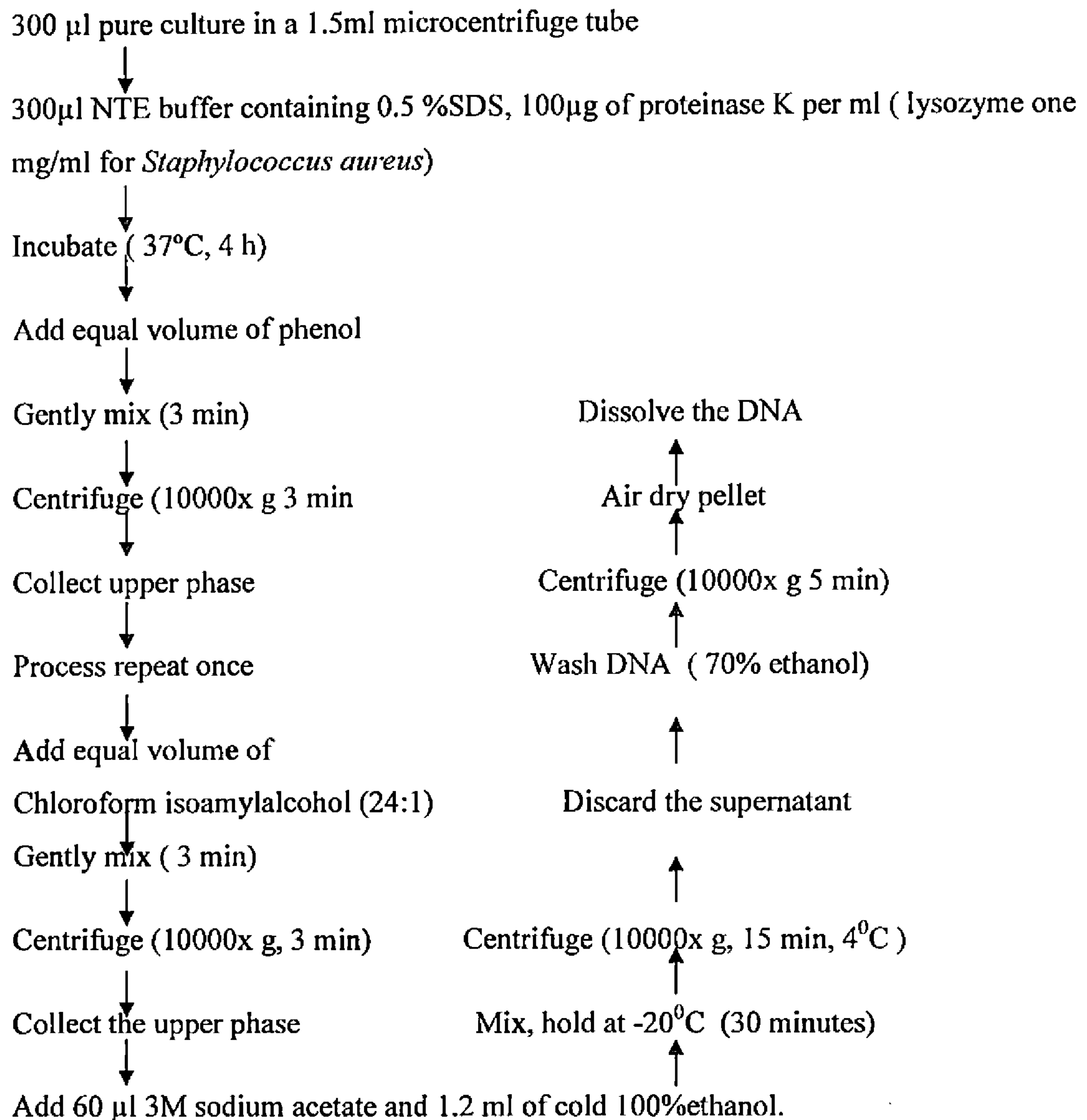
3.1.2.5 Sample (milk) preparation and phenol chloroform method of DNA extraction.

The procedure is a modification of Murphy *et al.* (2002). Milk (1.5ml) was taken in a 2ml microcentrifuge tube. To this 50mM EDTA was added and allowed to stand ten minutes. It was then centrifuged to remove the fat and supernatant was discarded. The pellet was washed with sterile PBS and resuspended in 100µl PBS.

The phenol chloroform extraction (Phuektes *et al.*, 2001) was followed with modifications. The temperature of incubation was increased from 37°C to 80 °C and time was reduced from four hours to 10 minutes (Flow Chart 3.).

The extracted DNA was detected by electrophoresis in 0.7 per cent agarose gel in TAE buffer (1X). Agarose was dissolved in TAE buffer (1X) by heating and ethidium bromide was added to a final concentration of 0.5 µg/ml. The comb was kept in proper position, and melted agarose was then poured into clean, dry, gel platform. Once the gel was set, the comb was removed gently and the tray containing the gel was completely immersed in TAE buffer. The extracted DNA (1 µl) was mixed with one µl of 6X gel loading buffer and the samples were loaded in the wells. DNA ladder (100 bp) was used as marker. Electrophoresis was carried at 70V for

Flow chart 2. Preparation of template DNA from pure culture of gram negative bacteria (*Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus*) by phenol chloroform extraction (Phuektes *et al.* 2001)



half an hour or until the bromophenol blue dye migrated to more than two-third of the length of the gel. The gel was visualized under UV transilluminator (UVITEC, UK) and the images were documented in a gel documentation system (UVITEC, UK).

3.2 POLYMERASE CHAIN REACTION

3.2.1 Materials

3.2.1.1 PCR reaction buffer (10X)

This included 1M KCl, 100mM Tris,15 mM $MgCl_2$, 500μg/ml BSA (bovine serum albumin) and 0.5% Tween 20

3.2.1.2 Triple distilled water

3.2.1.3 Deoxyribo Nucleotide Triphosphate (dNTP) mix (Bangalore Genei)

10mM (2.5mM of each dGTP, dCTP, dATP and dTTP in equal volume.

3.2.1.4 Magnesium chloride 25mM (Bangalore Genei)

3.2.1.5 Taq DNA polymerase (Bangalore Genei)

Taq DNA polymerase enzyme with a concentration of 3U/μl.

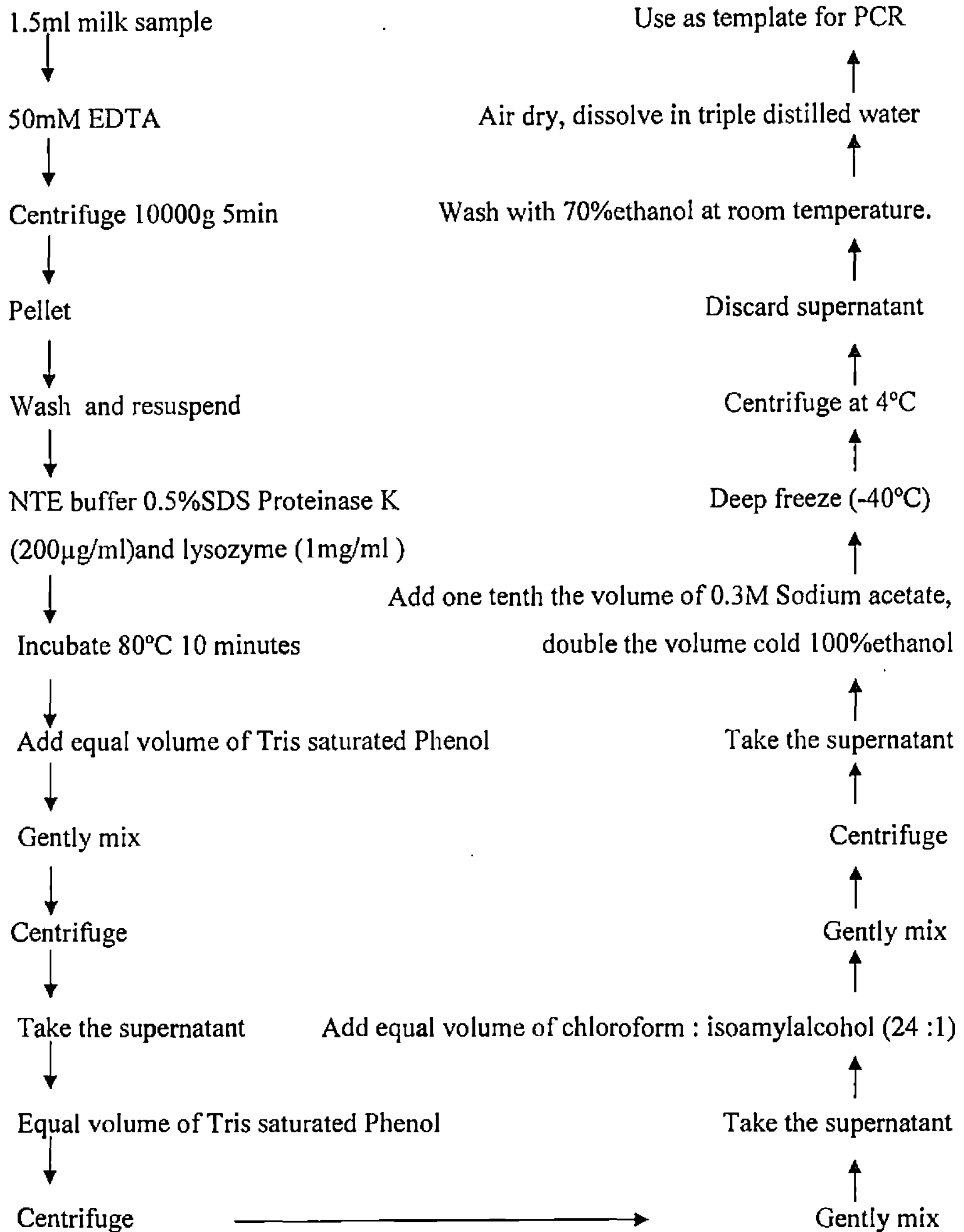
3.2.1.6 Primers (Bangalore Genei)

Specific primers to detect Pseudomonas species designed by Spilker *et al.* (2004) were used. The sequences of the primers were as follows:

Forward 5'- GACGGGTGAGTAATGCCTA-3'

Reverse 5'-CACTGGTGTTCCTTCCTATA -3'

Flow chart 3. Extraction of DNA extraction from milk



was added and mixed well to form the stock and this was diluted to one in ten as described above. These reconstituted solutions containing 10pm/μl primers each were used in PCR.

Pseudomonas aeruginosa

The tubes containing lyophilized primers were centrifuged at 10,000g for five minutes in a cooling centrifuge. To the tube containing forward primer (15,789 picomoles), 158 μl of triple distilled water was added and mixed well to form the stock. From this,10 μl was taken and 90 μl of triple distilled water was added. To the tube containing reverse primer (15,789 picomoles) 158 μl of triple distilled water was added and mixed well to form the stock and this was diluted to one in ten as described above. These reconstituted solutions containing 10pm/μl primers each were used in PCR.

Escherichia coli

The tubes containing lyophilized primers were centrifuged at 10,000g for five minutes in a cooling centrifuge. To the tube containing forward primer (15,789 picomoles), 158 μl of triple distilled water was added and mixed well to form the stock. From this, 10 μl was taken and 90 μl of triple distilled water was added. To the tube containing reverse primer 15,789 picomoles) 158 μl of triple distilled water was added and mixed well to form the stock and this was diluted to one in ten as described above. These reconstituted solutions containing 10pm/μl primers each were used in PCR.

Staphylococcus aureus

The tubes containing lyophilized primers were centrifuged at 10,000g for five minutes in a cooling centrifuge. To the tube containing forward primer (15,000 picomoles), 150 μl of triple distilled water was added and mixed well to form the

stock. From this 10 μl was taken and 90 μl of triple distilled water was added. To the tube containing reverse primer (14,285 picomoles), 143 μl of triple distilled water was added and mixed well to form the stock and this was diluted to one in ten as described above. These reconstituted solutions containing 10pm/μl primers each were used in PCR.

3.2.2.1 Preparation of master mix

For PCR standardization chromosomal DNA from pure cultures of *P. aeruginosa, E. coli* and *S. aureus* were used.

PCR was performed in a total volume of 10μl reaction mixture. A master mix was prepared before setting up the PCR reaction by combining the following reagents in a 80μl volume.

Preparation of 80μl master mix for 10 reactions

PCR reaction buffer	10 μl
Forward primer	5 μl
Reverse primer	5 μl
dNTPmix	8 μl
$MgCl_2$	10 μl
Taq DNA polymerase	2 μl
Triple distilled water	40 μl

To each PCR tube, 2.0 μl of template DNA and 8.0 μl of master mix were added. One negative control without DNA was also added. The PCR amplification

was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme.

3.2.2.2 Programme of amplification for Pseudomonas aeruginosa

One cycle	Denaturation	95°C	Two minutes
25 cycles	Denaturation	94°C	20 seconds
	Annealing	58°C	20 seconds
	Extension	72°C	40 seconds
One cycle	Extension	72°C	one minute

3.2.2.3 Programme of amplification for Pseudomonas species

One cycle	Denaturation	95°C	Two minutes
25 cycles	Denaturation	94°C	20 seconds
	Annealing	54°C	20 seconds
	Extension	72°C	40 seconds
One cycle	Extension	72°C	one minute

3.2.2.4 Programme of amplification for Escherichia coli

One cycle	Denaturation	95°C	Six minutes
35 cycles	Denaturation	95°C	20 seconds
	Annealing	72°C	20 seconds
	Extension	72°C	20 seconds
One cycle	Extension	72°C	Five minute

3.2.2.5 Programme of amplification for Staphylococcus aureus

One cycle	Denaturation	95°C	Five minutes
35 cycles	Denaturation	95°C	One minute
	Annealing	54°C	30 seconds
	Extension	72°C	30 seconds
One cycle	Extension	72°C	Seven minutes

All the reactions were conducted under the heated lid at 104°C. The product was analyzed by submarine agarose gel electrophoresis.

3.2.3 Submarine agarose gel electrophoresis

3.2.3.1 Materials

A. (0.5 M) EDTA (pH 8.0)

EDTA (18.61g) was dissolved in about 95ml triple distilled water and pH was adjusted to 8.0 with sodium hydroxide crystals to prepare a stock solution of 0.5M EDTA (pH 8.0)

B. TAE (Tris-Acetate EDTA) buffer (50X) pH 8.0

Tris base	48.40 g
Glacial acetic acid	11.42 ml
0.5 M EDTA pH 8.0	20.00 ml
Distilled water to	1000 ml

Autoclaved at 121°C for 15 minutes at 15 lbs pressure and stored at room temperature.

C. TAE (1X)

TAE 50X	2.00 ml
Distilled water	98.00 ml

D. Agarose Gel (1.0 per cent)

Agarose low EEO (Genei)	0.5g
TAE buffer (1X)	50.0 ml

E. Gel loading buffer (6X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Sucrose	40.00 g
Distilled water to	100.00 ml

Stored at 4°C.

F. Ethidium bromide

Ethidium bromide	100 mg
Distilled water	10 ml

Stored at 4°C in amber coloured bottles.

G. DNA molecular size marker

The 100bp molecular size markers were obtained from Bangalore Genei (India).

3.2.3.2 Method

The PCR product was detected by electrophoresis in 1.0 per cent agarose gel in TAE buffer (1X). Agarose was dissolved in TAE buffer (1X) by heating and ethidium bromide was added to a final concentration of 0.5 μg/ml. Melted agarose was then poured into clean, dry, gel platform, and the comb was kept in proper position. Once the gel was set, the comb was removed gently and the tray containing the gel was completely immersed in TAE buffer. Amplified PCR product (5.0 μl) was mixed with one μl of 6X gel loading buffer and the samples were loaded in the wells. A 100 bp DNA ladder was used as marker. Electrophoresis was carried at 70V for one hour (or) until the bromophenol blue dye migrated to more than two-third of the length of the gel. The gel was visualized under UV transilluminator (UVITEC, UK) and the images were documented in a gel documentation system (UVITEC, UK).

3.3 SENSITIVITY OF PCR

3.3.1 Materials

Sterile Phosphate Buffered Saline (HiMedia, Mumbai.)
Brain Heart Infusion agar (HiMedia, Mumbai.)
Bacterial pure culture (MTCC, Chandigarh.)

3.3.2 Method

Pure culture of *P. aeruginosa,* was streaked on to brain heart infusion agar and incubated overnight at 37°C. A few colonies were suspended in 2ml PBS, centrifuged and the pellet resuspended in PBS to make a concentration of 10^8 organisms per millilitre with McFerland standard. Then serial dilutions were made in PBS upto 10 organisms per millilitre. The same procedure was repeated for *E. coli* and *S. aureus.* DNA extraction was done as by the phenol chloroform method. PCR was done as

above. The minimum detection levels of pathogens were determined both in PBS as well as in milk.

3. 4 SPECIFICITY OF PCR

DNA extraction was done as by the phenol chloroform method from culture which contained *E. coli, S. aureus* and *P. aeruginosa*. The DNA obtained was used as template and individual PCR was done with each of the primers specific for *E. coli, S. aureus* and *P. aeruginosa*. This was done to check the specificity of the primers to detect the specific pathogen.

3. 5 CULTURE METHOD

3.5.1 Aerobic Plate Count

Aerobic Plate Count (APC) of each sample was estimated by pour plate technique, as described by Mortan (2001). From the selected 10 fold dilution of each sample, one ml of the inoculum was transferred to duplicate petridishes of uniform size. To each of the inoculated plates about 15-20 ml sterile molten standard plate count agar maintained at 45°C was poured and mixed with the inoculum, by gentle rotary movement clock wise, anticlock wise, forward and backward. The inoculated plates were left at room temperature and allowed to solidify, and incubated at 37°C for 24h. At the end of incubation plates showing between 30 and 300 colonies were selected and counts were taken with the help of a colony counter and expressed in colony forming units (cfu) per ml.

3.5.2 Pseudomonas

For the isolation of Pseudomonas, a loopful of the sample was streaked on to duplicate plates of Pseudomonas Agar Base (Hi-media) supplemented with Cetrinix (FD 029, Hi-media) and plates were incubated at 30°C for 24 h (Cousin *et al.*, 2001).

172819

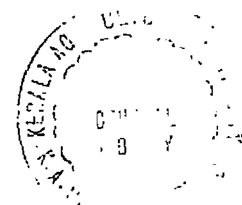
At the end of incubation, pigmented or non-pigmented smooth circular colonies were transferred to nutrient agar slants and incubated at 30°C overnight and were stored at refrigeration temperature. The isolates were subjected to further characterization and identification by cultural, morphological and biochemical reactions described by Barrow and Feltham (1993).

3.5.3 *Escherichia coli*

For the isolation of *E. coli*, a loopful of inoculum from each sample was inoculated on to duplicate plates of HiCrome E.coli Agar and incubated at 37°C for 24h. (Indian Standards, 1980). At the end of incubation period, blue coloured colonies were selected and transferred on to nutrient agar slants and incubated at 37°C for overnight. These isolates were subjected to further characterization and identification by cultural, morphological and biochemical reactions as described by Barrow and Feltham (1993).

3.5.4 *Staphylococcus aureus*

For the isolation of *S. aureus*, a loopful of the sample was inoculated onto Baird-Parker (BP) agar medium (Hi-media) and was incubated at 37°C for 48 h. (Lancette and Bennett, 2001). At the end of incubation, colonies showing characteristics appearance (circular, smooth, convex, moist, 2.3 mm in diameter on uncrowded plates, gray black to jet black, frequently with light coloured margin, surrounded by opaque zone and frequently with outer clear zone) on BP agar medium were selected and transferred to nutrient agar slants and incubated at 37°C for overnight. The isolates were stored at refrigeration temperature. Characterization and identification of the isolates were done following the procedure described by Barrow and Feltham (1993).



Results

4. RESULTS

In the present study polymerase chain reaction was applied for the rapid evaluation of hygienic quality of milk. Phenol chloroform method of DNA extraction was standardized for DNA extraction directly from milk. With modifications of time and temperature of incubation, the time required for DNA extraction could be reduced to within two and half hours. Polymerase chain reaction (PCR) was standardized to asses the bacterial load in milk using primers specific for the predominant milk spoilage organism Pseudomonas. Estimation of the bacterial count by PCR was compared with conventional culture method. The PCR was employed with specific primers to detect the presence of *Escherichia coli* and *Staphylococcus aureus* directly from milk. Specificity of primers was confirmed. Sensitivity of PCR was evaluated by serial dilution of the organisms in sterile chilled phosphate buffered saline (PBS) and also in milk. Then 50 milk samples were evaluated by PCR based assay for the bacterial load, detection of *E. coli and S. aureus,* which were then compared with conventional culture.

4.1 STANDARDIZATION OF DNA EXTRACTION

The predominant spoilage organism in milk was found to be Pseudomonas species. The Pseudomonas count by culture method ranged from 10^2-10^3 cfu /ml in raw milk. Hence pseudomonas was selected as the organism to assess the bacterial load by PCR.

4.1.1 Extraction of DNA from pure culture of Pseudomonas, *Escherichia coli and S. aureus* by boiling lysis and phenol chloroform method.

The DNA was successfully extracted from pure culture of *P. aeruginosa* and *E. coli* by boiling lysis method. The time required for DNA extraction by boiling lysis is less than one and half hours. Presence of DNA was confirmed by agarose gel

electrophoresis. DNA could be extracted from pure culture of *S. aureus* only by phenol chloroform method with the addition of lysozyme at the rate of one mg/ml along with NTE buffer. The time required for DNA extraction by phenol chloroform method was two hours.

4.1.2 Protocol standardization for DNA extraction of Pseudomonas, *Escherichia coli* and *Staphylococcus aureus* directly from milk

The DNA extraction directly from milk was unsuccessful by boiling lysis. Hence, phenol chloroform method of DNA extraction with modifications was standardized for DNA extraction directly from milk. The presence of DNA was confirmed by the presence of band on agarose gel electrophoresis. Lysozyme was required for the extraction of DNA from *S. aureus*.

DNA was extracted from milk by the phenol chloroform method of Phuektes *et al.* 2001. But the DNA obtained could not be amplified to get a PCR product using either *P. aeruginosa* or Pseudomonas species primer. But the DNA obtained from culture by the same method amplified to give PCR product of 618 bp with Pseudomonas species primer and 956 bp for *P. aeruginosa* primer (Fig.1).

The procedure for DNA extraction directly from milk was therefore modified with sample preparation to reduce the effect of inhibitors of PCR. Milk was treated with 50mM EDTA to chelate the calcium. Sample was centrifuged at 10,000g for 10 minutes to remove the fat and isolate the bacterial cells. The pelleted cells were resuspended and an equal volume of NTE buffer containing 0.5%SDS and 200 microgram (µg) proteinase K was added. Sample was incubated at 80°C for 10 minutes. This was followed by phenol chloroform method of DNA extraction. Presence of DNA was confirmed by agarose gel electrophoresis. The extracted DNA was used as template for PCR. The time for DNA extraction was less than two and half hours.

Fig 1. *Pseudomonas aeruginosa.*

Lane 1 & 12. 100bp DNA ladder
Lane 2 - 11. PCR product (956bp) obtained from pure culture of *P. aeruginosa.*

4.1.2.1 Detection of E. coli in milk using specific primers

The DNA obtained from pure culture of *E. coli.* by boiling lysis method was amplified by *E. coli* specific primers (Daly *et al.* 2002) to obtain a 366 bp PCR product.

PCR product was obtained from template DNA extracted directly from milk, when sample preparation and phenol chloroform extraction was done as mentioned above Pseudomonas.

PCR product was obtained when the culture was incubated at 80°C /10 minutes, 80°C / 30 minutes, 50°C / half an hour, 50°C / one hour, 50°C / one and half hours, 50°C two hours, 50°C / two and half hours, 50°C / three hours, 37°C / four hours and 37°C overnight (Fig.2).

4.1.2.2 Detection of S. aureus in milk using specific primers

The DNA could not be obtained from pure culture of *S. aureus* by boiling lysis. *S. aureus* DNA could successfully be isolated by the phenol chloroform method with the addition of lysozyme at the rate one mg/ml along with NTE buffer. The DNA used as template in PCR was amplified to give a product of 420 bp.

4.2 STANDARDIZATION OF PCR

The reaction mixture contained PCR buffer, triple distilled water, magnesium chloride, forward and reverse primers dNTP mix Taq DNA polymerase and template DNA in a 10 µl reaction.

4.2.1 Template

The PCR was done in 10 µl reaction. Template (obtained from pure culture) concentrations of 0.5, 1.0, 2.0, 3.0 and 4.0 µl per reaction were used in PCR. The

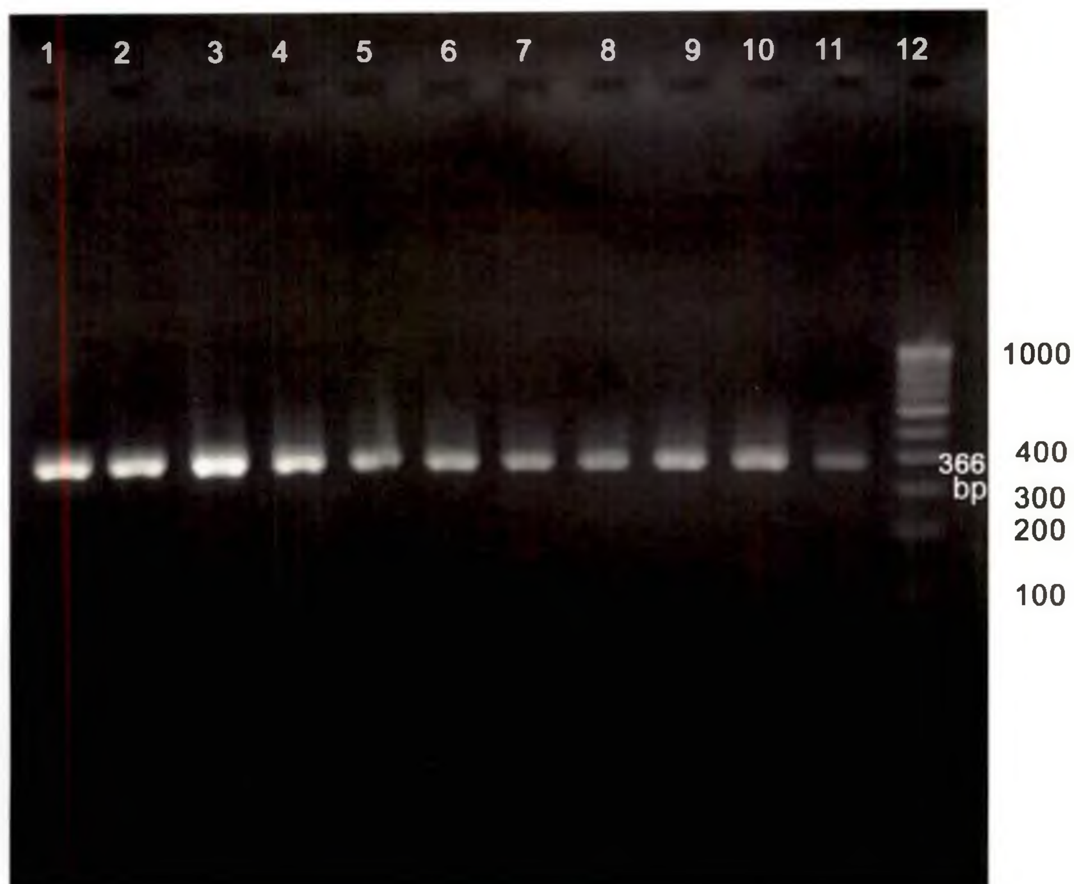


Fig. 2. PCR for E.coli using template DNA extracted using various temperatures of incubation.

Lane 1. 80°C / 10min
Lane 2. 80°C / 30min
Lane 3. 50°C / 30min
Lane 4. 50°C / 1h
Lane 5. 50°C / 1h 30min
Lane 6. 50°C / 2h
Lane 7. 50°C / 2h
Lane 8. 50°C / 3h
Lane 9. 37°C / 4h
Lane 10. 37°C / overnight
Lane 11. Positive control
Lane 12. DNA ladder

PCR product was obtained for all reactions along with non specific amplification when 4.0 µl of template was used.

PCR was done in 10 µl reaction. PCR was done with template (obtained directly from milk) concentrations of 1.0 µl, 2.0 µl and 3.0 µl per reaction. PCR product was obtained for all reactions.

4.2.2 PCR reaction buffer (10X)

For template DNA from pure culture PCR products were obtained when PCR 10x buffer containing 500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$ with addition of 1mM $MgCl_2$ or on using the buffer containing1M KCl, 100 mM Tris, 15 mM $MgCl_2$. 500 µg/ml BSA and 0.5% Tween 20 with addition of 1mM $MgCl_2$ or Magnesium free PCR 10x buffer with addition of 2.5 mM $MgCl_2$.

For template DNA isolated directly from milk PCR products were obtained only when PCR 10x buffer containing1M KCl, 100 mM Tris, 15 mM $MgCl_2$, 500 µg/ml BSA and 0.5% Tween 20 with addition of 1mM $MgCl_2$. PCR product could not be obtained when PCR 10X buffer containing 500 mMKCl, 100 mM Tris-HCl, 15 mM $MgCl_2$ with addition of 1mM $MgCl_2$ or when using Magnesium free PCR 10X buffer with addition of 2.5 mM $MgCl_2$.

4.2.3 Taq DNA polymerase

For template DNA from pure culture PCR products were obtained when concentration of Taq was 0.3 U per 10 µl reaction.

For template DNA isolated directly from milk PCR products were obtained when concentration of Taq was 0.6 U per 10 µl reaction.

4.2.4 Annealing temperature

The optimal annealing temperature for Pseudomonas species, *E. coli* and *S. aureus* was 54°C, 72°C and 54°C respectively.

4.2.5 Product of PCR

For Pseudomonas the amplified PCR product was 618 bp. For *Escherichia coli* the amplified product was 326 bp DNA fragment. A DNA fragment of 420 bp was amplified by the PCR for *Staphylococcus aureus.*

4.2.6 Time required for PCR

The time taken for PCR of Pseudomonas and *E. coli* was one hour and 15 minutes, for *S. aureus was* one hour and half hours. The required for agarose gel electrophoresis was one hour. Therefore, the total time required for DNA extraction, PCR and electrophoresis was approximately less than five hours (Table 1.).

4.3 DETERMINATION OF SENSITIVITY OF PCR ASSAY

The sensitivity of PCR assay to determine the limit of detection of Pseudomonas (Fig. 3), *Escherichia coli* (Fig. 4) and *Staphylococcus aureus* (Fig. 5) was checked both in PBS (Table 2.) and milk (Table 3.). The level of sensitivity of PCR assay was higher for DNA extracted from PBS compared to that from milk for *S. aureus*. *P. aeruginosa, E. coli* and *S. aureus* were spiked individually in phosphate buffered saline, to get concentrations ranging from 10^8 to 10 organisms per ml. DNA was extracted from the different concentrations of organisms spiked, which were used as template for PCR. The lowest detection level of Pseudomonas and *E. coli* was 10 organisms per ml both in PBS and milk. But for *S. aureus*, the lowest detectable level was 10^3 organisms per ml in PBS and 10^4 organisms per ml in milk.

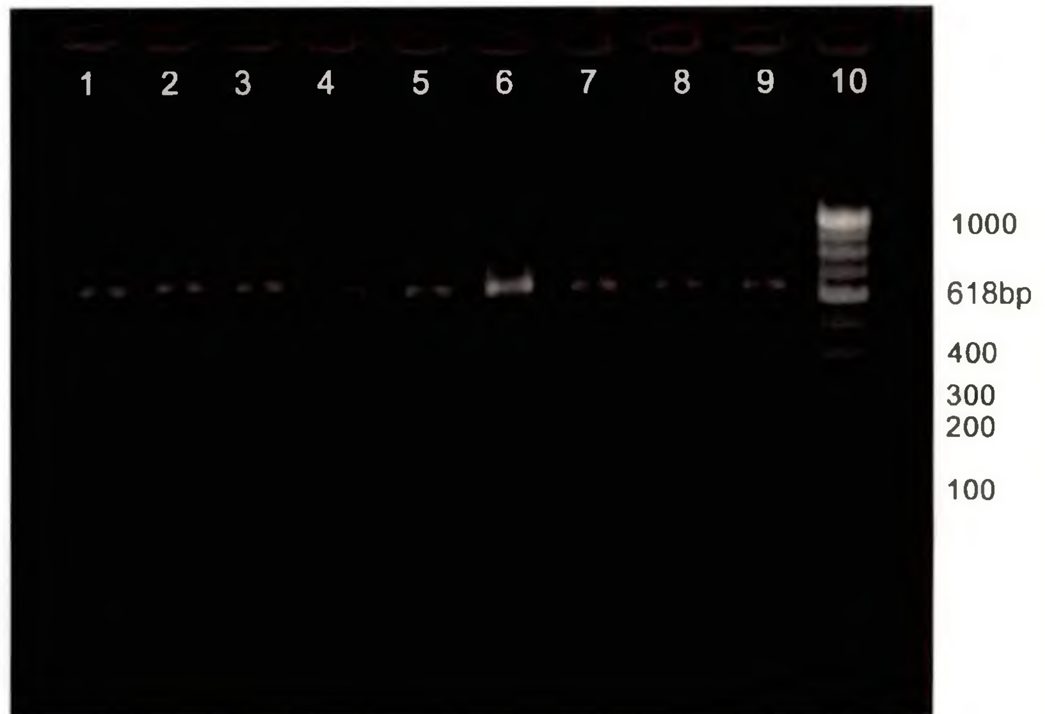


Fig 3. Sensitivity of PCR for Pseudomonas species.

Lane 1-8. **Concentrations of 10^8 - 10 organisms per ml of milk**
Lane 9. **Positive control**
Lane 10. **100bp DNA ladder**

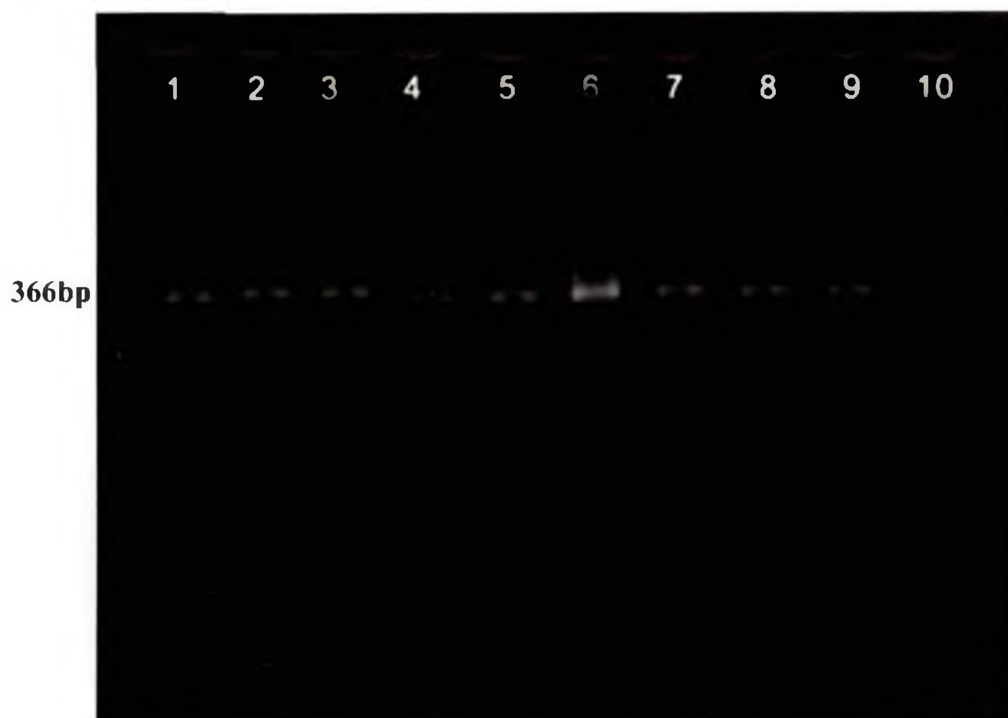


Fig 4. Sensitivity of PCR for *E. coli*

Lane 1 - 8. **Concentrations of 10^8 - 10 organisms per ml of milk**
Lane 9. **Positive control**
Lane 10. **100bp DNA ladder**

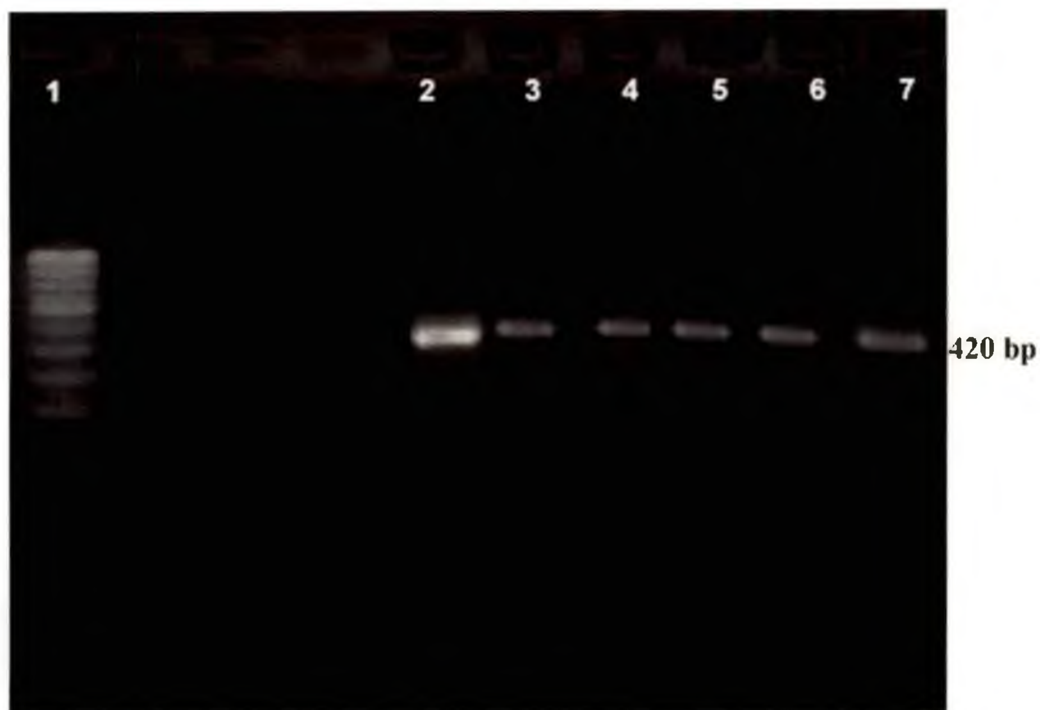


Fig 5. Sensitivity of PCR for *Staphylococcus aureus.*

Lane 1. **100bp DNA Ladder**
Lane 2. **Positive control**
Lane 3 - 7. **10^4 - 10^8 Organisms per ml of milk.**

Table 1. Comparison of time taken by different DNA extraction procedures

Reference	Method of DNA extraction	Time required		
		DNA extraction	PCR	time
Allmann *et al.* (1995)	digestion buffer	Six hours	Two hours	Nine hours
Phuektes *et al.* (1999)	Phenol chloroform	Six hours	One hour 30min	Eight hours 30 min
Daly *et al* (2002)	Alkaline extraction	Two hours 30min	One hour 30 min	Five hours
Ramesh *et al* (2002)	Chemical extraction	Five hours	Two hours	Eight hours
Gupta et al (2006)	Phenol chloroform	Two hours 30min	Two hours	Five hours 30min
McKillip *et al*	Guanidinium isothiocyanate	Two hours 30min	Two hours	Five hours 30min
Present study	Phenol chloroform	Two hours 30min	One hour 30min	Five hours

Table 2. Detection level of pathogens by PCR in PBS

Organisms per ml (in PBS)	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Pseudomonas	+	+	+	+	+	+	+	+
Escherichia coli	+	+	+	+	+	+	+	+
Staphylococcus aureus	+	+	+	+	+	+	_	_

Table 3. Detection level of pathogens by PCR in milk

Organisms per ml (in milk)	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Pseudomonas	+	+	+	+	+	+	+	+
Escherichia coli	+	+	+	+	+	+	+	+
Staphylococcus aureus	+	+	+	+	+	_	_	_

4.4 SPECIFICITY OF PRIMERS

None of the primer pairs yielded products with DNA from other bacterial species. Only the DNA of *E. coli* was amplified when primers specific for *alr* gene of *E. coli* was used. When the oligonucleotide primers from the 16S to 23S rRNA intergenic spacer region for *S. aureus* was used only the DNA of *S. aureus* was

amplified. The primers for Pseudomonas amplified only the DNA from Pseudomonas species.

4.5 ANALYSIS OF MILK SAMPLES BY PCR AND CONVENTIONAL CULTURE METHOD

4.5.1 Estimation of bacterial load by PCR and culture method

Milk was serially diluted upto 10^{-10}dilutions and DNA was extracted by the method standardized. The extracted DNA was used as template for PCR assay. PCR was carried out with primers specific for Pseudomonas species. Amplification of 618bp PCR product was observed upto 10^{-7} dilution of milk samples. The APC as estimated by the culture method was 10^{6}cfu/ml (Fig. 6.). It could be concluded that the approximate APC of the milk sample by PCR is next lower dilution to the dilution giving the PCR amplification (Table 4.).

Table 4. Estimation of bacterial load by PCR and conventional culture method.

Dilution	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Culture method (APC) cfu/ml	30-300	<30	<30	–
PCR	+	+	+	+

(n=50)

4.5.2 Detection of pathogens in milk by PCR and conventional culture method

Culture was done for milk samples for the isolation of *E. coli* and *S. aureus*.

Fig 6. Assessment of bacterial load in milk based on Pseudomonas species

Lane 1. Blank
Lane 2. 10^{-1} dilution
Lane 3-8. 10^{-2} to 10^{-7} dilution
Lane 9. Positive control
Lane 10. Negative control
Lane 11. 100bp DNA ladder

4.5.2.1 Escherichia coli

For the isolation of *Escherichia coli*, a loopful of inoculum from each sample was inoculated on to duplicate plates of HiCrome Ecoli agar and incubated at 37°C for 24 h. At the end of incubation period, three or four blue coloured colonies (Plate 1.) were selected and transferred on to nutrient agar slants and incubated at 37°C for overnight. These isolates were subjected to further characterization and identification by cultural, morphological and biochemical reactions. Extraction of DNA and PCR was done with primers for detection of *E. coli* from the same milk samples and compared with culture. Percentage of samples positive both by culture and PCR was 50 and negative by both methods were 30. Twenty percentage of the samples were positive by PCR and negative by culture (Table 5.).

Table 5. Comparative assessment of detection of *E. coli* in milk by PCR and conventional culture method.

Percentage of samples	*Escherichia coli*	
	PCR	Culture
50	+	+
30	–	–
20	+	–

4.5.2.2 Staphylococcus aureus

For the isolation of *S. aureus*, a loopful of the sample was inoculated onto Baird-Parker agar medium and was incubated at 37°C for 48 h. At the end of

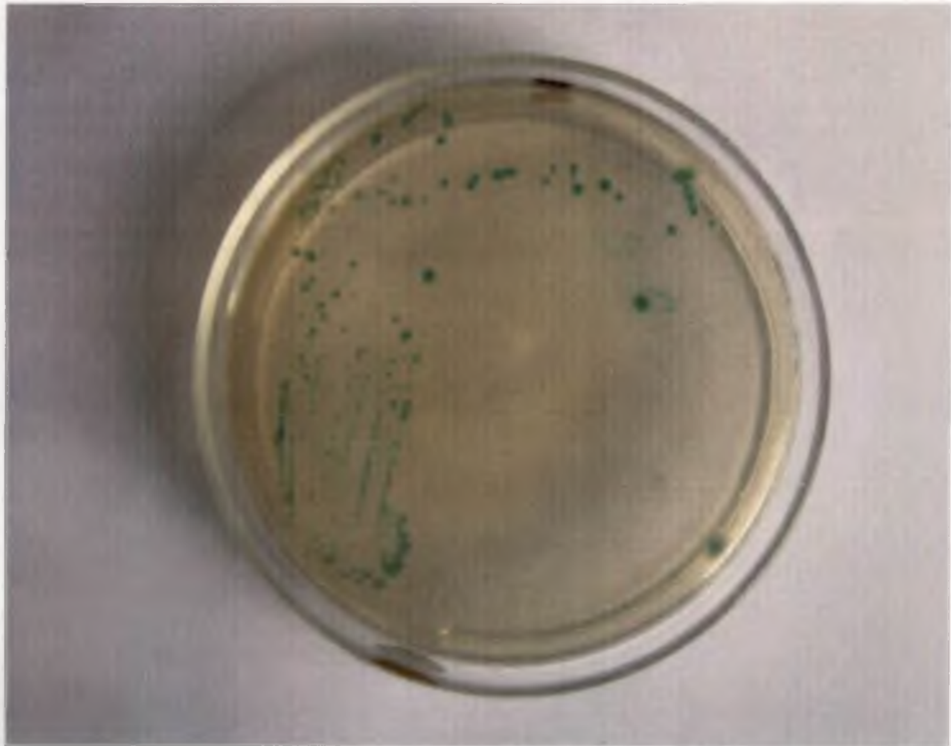


Plate 1. ***Escherichia coli*** **in Hicrome E.coli Agar**

Plate 2. ***Staphylococcus aureus*** **in Baird - Parker Agar**

incubation, colonies showing characteristics appearance on BP agar medium (Plate 2.) were selected and transferred to nutrient agar slants and incubated at 37°C for overnight. These isolates were subjected to further characterization and identification by cultural, morphological and biochemical reactions. Extraction of DNA and PCR was done with primers for detection of *S. aureus* from the same milk samples and compared with culture. Percentage of samples positive both by culture and PCR was 60 and negative by both methods were 20. Twenty percentage of the samples were negative by PCR and positive by culture (Table 6.).

Table 6. Comparative assessment of detection of *S. aureus* in milk by PCR and conventional culture method.

Percentage of samples	*Staphylococcus aureus*	
	PCR	Culture
60	+	+
20	–	–
20	–	+

Discussion

5. DISCUSSION

Unhygienic production of milk leads to reduced shelf life, rapid microbial growth and multiplication which pose a potential health hazard to the consumers. Microbial food safety gaining major public health concern worldwide makes it necessary for the rapid detection foodborne pathogens as well as quick assessment of hygienic status. Various molecular based techniques have been used as useful tools to detect the foodborne pathogens (Winters, 1998). In the present study polymerase chain reaction was applied for rapid evaluation of hygienic quality of milk. Phenol chloroform method of DNA extraction was standardized for DNA extraction directly from milk. With modifications of time and temperature of incubation the time for DNA extraction could be reduced to within two hours. Polymerase chain reaction was standardized to asses the bacterial load in milk using primers specific for the predominant milk spoilage organism Pseudomonas. Estimation of the bacterial count by PCR was compared with conventional culture method. The PCR was employed with specific primers to detect the presence of *Escherichia coli* and *Staphylococcus aureus* directly from milk. Specificity of primers was confirmed. Sensitivity of PCR was evaluated by serial dilution of the organisms in sterile chilled PBS and also in milk. Then 50 milk samples were evaluated by PCR based assay for the bacterial load, detection of *Escherichia coli and S. aureus,* which were then compared with conventional culture.

In the present study, the boiling lysis method of DNA extraction could isolate DNA only from pure culture of *Pseudomonas aeruginosa* and *Escherichia coli* but not from *Staphylococcus aureus.* DNA from *S. aureus* could not be extracted by the boiling lysis method probably due to the robustness of gram positive cells. The use of lysozyme for efficient release of DNA has been earlier reported by Meiri-Bendek *et al.* 2002. The time for DNA extraction by boiling lysis is less than one and half hours.

The boiling lysis method done for culture failed to extract DNA from milk. The presence of milk fat and proteins interfered with the lysis of bacterial cells. Hence, the phenol chloroform method (Phuektes *et al.* 2001) was adopted for DNA extraction directly from milk. This method could extract DNA from culture and milk. But it took more than five hours for DNA extraction without enrichment. Although the DNA obtained from culture by this method produced amplified product by PCR, that from milk failed to give the product. Phuektes *et al.* (2001) reported that this method of DNA extraction was less sensitive than culture without enrichment. Therefore, a prior sample preparation had to be adopted to reduce the effect of PCR inhibitors and modified to reduce the time required for DNA extraction.

Sample preparation serves several functions for PCR detection (Lantz *et al.*, 2000). It initially decreases sample volume and concentrates the PCR template into a workable volume. In case of food samples it is necessary to remove inhibitors of PCR and concentrate the pathogens from food matrix. Therefore, efficient sample preparation is necessary. In the present study, the sample is milk which is found to be inhibitory due to food matrix itself (Rossen *et al.*,1992) and components such as calcium ions, proteinase, fats and milk proteins may shield access to the DNA polymerase (Wilson, 1997). Casein interferes with the proteinase K digestion of enzymes that degrade the somatic cell DNA (Murphy *et al.*, 2002). Milk was treated with 50mM EDTA so as to dissolve the caseine. Caseine requires calcium to form micelles (Holt, 1992). When EDTA is added to milk it chelates and removes calcium from caseine, therefore caseine dissolves. On further centrifugation the pellet at the bottom is free of caseine.

The time for DNA extraction was reduced to less than two and half hours. The temperature of incubation was raised from 37°C to 80°C and time reduced from four hours to 10 minutes. The maximum activity of Proteinase K is 65°C and activity is

reduced with increasing temperatures and completely inactivated at 95°C for 15 minutes (Kuzma *et al.* 2003). The concentration of proteinase K was therefore increased from 100µg per ml to 200µg per ml in the present study. The time for DNA extraction was two and half hours. The phenol chloroform method of DNA extraction method took six hours (Phuektes *et al.*, 2001) and four hours (Romero and Lopez-Goni, 1999).

PCR was conducted in a 10 µl PCR reaction mixture. Amplified product was obtained when the template (obtained from culture) concentration was 0.5 µl, 1.0 µl, 2.0 µl and 3.0 µl; beyond which there were non specific amplifications. For DNA from milk, amplification product was obtained for 1.0 µl, 2.0 µl and 3.0 µl concentration beyond which nonspecific amplification or no product was obtained in a 10 µl reaction. Kuzma *et al.* (2003) got amplified PCR product from template concentrations 0.25 µl to 5.0 µl in a 50 µl reaction, beyond which no amplified product was obtained. This might be due to the fact that their samples were of intramammary infections where the count of somatic cells and pathogens are very high, yielding more DNA on extraction. So the volume of template required per reaction would be lesser compared with our procedure.

PCR product was obtained with 10x PCR buffer containing 500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$ with addition of 1 mM $MgCl_2$ or 10x standard PCR buffer for the template DNA obtained from pure culture. However, there was no amplification when template used was the one obtained from DNA extracted directly from milk. When 10 x PCR buffer containing 1 M KCl, 100 mM Tris, 15 mM $MgCl_2$ 500 µg/ml BSA and 0.5% Tween 20 with addition of 1mM $MgCl_2$ was used amplified product was obtained from template DNA of culture and that extracted directly from milk. Al- soud and Radstrom (2000) compared the effect of 16 amplification facilitators and found that BSA reduced the inhibition of both rTth and Taq DNA polymerase in the presence of all types of inhibitors (blood, faeces and

meat). The ability of gp32 to reduce the inhibition of Taq DNA polymerase by faeces was not reproducible. BSA was found to overcome the inhibition of Taq DNA polymerase by proteinases (Powell *et al.*, 1994), which are inhibitory in milk.

For template DNA from pure culture PCR, products were obtained when concentration of TAQ was 0.3 U per 10 µl reaction. For template from milk, product was obtained for Taq concentration 0.6U per reaction. In the present study 0.6U per reaction was used to overcome any inhibitory action of Taq polymerase inhibitors. The inhibitors interfere with the cell lysis necessary for extraction of DNA. They interfere by nucleic acid degradation or capture and they inhibit polymerase activity for amplification of target DNA (Wilson, 1997).

5.1 SPECIFICITY OF PCR

The primers were used in this study to identify the genus and species specific 16S rDNA signature sequences which were 100 per cent specific for *P. aeruginosa* (956 bp PCR product) and *Pseudomonas species* (618 bp PCR product) was reported by Spilker *et al.* (2004). The primers are highly specific and PCR product is produced only from the specific organism from culture which contained *P. aeruginosa, E. coli* and *S. aureus* and also from milk containing varied microflora.

The primer pairs amplified the DNA from *E. coli* to produce the expected PCR product of 366 bp. The primers specific for the conserved region within the alanine racemase gene was used by Daly *et al.* (2002). Alanine racemase is produced by all *E. coli* strains, including O157, and is essential for cell wall biosynthesis by providing D-alanine to peptidoglycan. Thus, detection of *alr* gene may be useful for the detection of *E. coli* in foods (Yokoigawa *et al.* 1999). The *alr* genes could detect a wide range of *E. coli* strains and also lead to detection of Shigella species but the incidence level of Shigella in foods is ordinarily very low (Gonzalez *et al.*, 1999).

The primers used by Phuektes *et al.* (2001) were used from the 16S to 23S rRNA intergenic spacer region for *S. aureus.* PCR product of expected size 420 bp was obtained from culture which contained *P. aeruginosa, E. coli* and *S. aureus* and milk containing varied microflora.

5.2 SENSITIVITY OF PCR ASSAY

The sensitivity of PCR assay to determine the limit of detection of Pseudomonas, *Escherichia coli* and *Staphylococcus aureus* was checked both in PBS and milk. The level of sensitivity of PCR assay was higher for DNA extracted from PBS compared to that from milk for *S. aureus.* Riffon *et al.* (2001) had reported a higher level of sensitivity for S. aureus detection using PCR in tryptic soy broth in milk. *Pseudomonas aeruginosa, E. coli* and *S. aureus* were spiked individually in phosphate buffered saline, and concentration of the organism was adjusted from10^8 to 10 organisms per ml. DNA was extracted from the different concentrations of organisms spiked which were used as template for PCR. The lowest detection level of Pseudomonas and *E. coli* was 10 organisms per millilitre both in PBS and milk. With efficient removal of PCR inhibitors by sample preparation by use of EDTA (Murphy *et al.* 2002) and centrifugation (Allmann *et al.*1995) and use of facilitators in PCR the detection limit in both PBS and milk were the same. Sensitivity of the DNA extraction procedure was found to achieve a detection limit of 10cfu/ml both in milk and sterilized saline (Cremonesi *et al.*, 2006). But for *S. aureus,* the lowest detectable level was 10^3 organisms per ml in PBS and 10^4 organisms per ml in milk. The gram positive cells having robust cell wall necessitate the use a combination of enzymes lysozyme, lysostaphin and lyticase and incubation conditions. (Riffon *et al.*, 2001 and Meiri-Bendek *et al.*, 2002).

5.3 ANALYSIS OF MILK SAMPLES BY PCR AND CONVENTIONAL CULTURE METHOD

Bacterial load of milk was assessed by PCR based on the presence of amplified product of Pseudomonas species, being the predominant milk spoilage organism. Amplified product was obtained upto 10^{-7} dilution of milk for which the aerobic plate count was 10^{6}cfu/ml. For sample with APC 10^{6}cfu/ml, amplified product was obtained upto 10^{-7} dilution. Both methods gave almost similar results PCR being less time consuming and there is chance of amplification from non viable or non culturable cells. This could explain the reason for PCR being positive where the culture was negative. Devatkal *et al.* (2005) employed the PCR technique to detect the bacterial load of buffalo liver stored aerobically at 4 ± 1°C. When PCR was carried out amplified product was obtained from dilutions of 10^{-1} to 10^{-4}. The total count by conventional culture was 24x 10^{4}cfu/g liver. Thus PCR and cultural methods gave similar results.

Percentage of samples positive for *E. coli* both by culture and PCR was 50 and negative by both methods were 30. Twenty percentage of the samples were positive by PCR and negative by culture. According to Hill (1996), a positive PCR result is not conclusive that viable cells are present in the sample, as dead cells contain amplifiable DNA. But the presence of dead cells too is of significance as it indicates that the product was not prepared from ingredients of sufficient quality or viable cells were present at the time food was consumed which poses a health hazard.

Percentage of samples positive *S. aureus* both by culture and PCR was 60 and negative by both methods were 20. Twenty percentage of the samples were negative by PCR and positive by culture. This could be due to inefficient DNA extraction, probably due to the low level of the organisms present in milk. Aslam *et al.* (2003), reported that when bacteria were added to milk and DNA was extracted immediately by rapid boil method the detection limit of PCR was as low as 5 cfu of bacteria per ml of milk or Phosphate Buffered Saline (PBS). The decrease in sensitivity of PCR detection when bacteria were grown overnight in milk was attributed to the coating of

bacteria with milk fat and consequent insufficient bacterial lysis. Romero and Lopez-Goni (1999) had also observed that improper DNA extraction could account for PCR negative results.

Summary

6. SUMMARY

The present study was conducted to develop a PCR based assay for the rapid evaluation of hygienic status of milk. For this the procedure for DNA extraction directly from milk was standardized. The extracted DNA was then used as template for PCR. The standardized procedure was thereafter applied for the assessment of bacterial load using primers specific for Pseudomonas which was compared with aerobic plate count by culture. PCR was also standardized for the detection of *Escherichia coli* and *Staphylococcus aureus* in milk.

First, DNA extraction was done from pure culture of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* by boiling lysis method. The procedure could not extract DNA from *S. aureus* and also failed to extract DNA from any organism when applied directly to milk. Therefore phenol chloroform method of extraction was applied to culture which yielded DNA from all organisms with the requirement of lysozyme for *S. aureus* for the complete release of DNA from lysed cells. The time required for DNA extraction was approximately six hours. This procedure when applied to milk, though yielded DNA could not be amplified by PCR.

The procedure was therefore modified to reduce the time required for DNA extraction and to remove the inhibitors of PCR in milk. For this prior sample preparation was done for removal of fat and casein. The time and temperature of incubation after addition of lysis buffer for the disruption of cells was changed to minimize the time required for DNA extraction. Thereby a reduction in the total time required for detection of pathogens was effected. Polymerase chain reaction was done with already published primers. Changes were made with inclusion of PCR buffer containing facilitators to overcome the effect of inhibitors of PCR when template used was that extracted directly from milk.

The lowest detection level of Pseudomonas and *E. coli* was 10 organisms per ml both in PBS and milk. But for *S. aureus*, the lowest detectable level was 10^3 organisms per millilitre in PBS and 10^4 organisms per millilitre in milk. The primers were highly specific and none of the primer pairs yielded products with DNA from other bacterial species. The total time taken for DNA extraction and PCR was five hours.

For the assessment of bacterial load, dilutions of milk upto 10^{-10} were made. DNA was extracted from all dilutions and PCR was conducted using primers for Pseudomonas species. Aerobic plate count was done with 10^{-5} to 10^{-7} dilutions. PCR amplification was obtained for dilution upto10^{-7} for samples with APC 10^6. For samples with APC 10^7 amplified product was obtained for dilution upto10^{-8}. Therefore it could be concluded that the approximate APC of the milk sample by PCR is next lower dilution to the dilution giving the PCR amplification.

Isolation of *E. coli* and *S. aureus* was done from milk by conventional culture method. Extraction of DNA and PCR was done with primers for detection of *E. coli* from the same milk samples and compared with culture. Percentage of samples positive both by culture and PCR was 50 and negative by both methods were 30. Twenty per cent of the samples were positive by PCR and negative by culture. Extraction of DNA and PCR was done with primers for detection of *S. aureus* from the same milk samples and compared with culture. Percentage of samples positive both by culture and PCR was 60 and negative by both methods were 20. Twenty per cent of the samples were negative by PCR and positive by culture.

References

7. REFERENCES

Aaku, E.N., Collison, E.K., Gashe, B.A. and Mapuchane, S. 2004. Microbiological quality of milk from two processing plants in Gaborone Botswana. *Food Control.* 15: 181-186

Adwan, G., Abu-Shanab, B. and Adwan, K. 2005. Enterotoxigenic *Staphylococcus aureus* in raw milk in the north of Palestine. *Turk. J. Biol.* 29: 229-232

Al Soud, W.A. and Radstrom, P. 2000. Effects of amplification facilitators on diagnostic PCR in presence of blood feces and meat. *J. Clin. Microbiol.* 38: 4463-4470.

Allmann, M., Hofelein, C., Koppel, E., Luthy, J., Meyer, R., Niederhauser, C., Wegmuller, B. and Candrian, U. 1995. Polymerase chain reaction (PCR) for detection of pathogenic microorganisms in bacteriological monitoring of dairy products. *Res. Microbiol.* 146: 85-97

Amagliani, G., Brandia, G., Omiccioli, E., Casiere, A., Brice, I.J. and Magnania, M. 2004. Direct detection of *Listeria monocytogenes* from milk by magnetic based DNA isolation and PCR. *Food Microbiol.* 21: 597–603

Arora, S., Agarwal, R.K. and Bist, B. 2006. Comparison of ELISA and PCR vis-a-vis cultural methods for detecting Aeromonas spp. in foods of animal origin. *Int. J. Food Microbiol.* 106: 177 – 183

Asha, K. 2007. Assessment of bacterial quality and shelf life of pasteurized milk. M.V.Sc. thesis, Kerala Agricultural University, Thrissur, p.174

Aslam, M., Hogan, J. and Smith, K.L. 2003. Development of a PCR-based assay to detect shiga toxin-producing *Escherichia coli, Listeria monocytogenes,* and Salmonella in milk. *Food Microbiol.* 20: 345–350

Barrow, C.J. and Feltham, R.K.A. 1993. *Cowan and Steel's manual for the Identification of Medical Bacteria.* Third edition. Cambridge Press, London, p.238

Barry, T., Colleran, G., Giennon, M., Dunican, L.K. and Gannon, F. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Appl.* 1: 51-56

Bej, A.K., Steffan, R.J., Dicesare, J., Haff, L. and Atlas, R.M. 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. *Appl. Environ. Microbiol.* 56: 307-314

Birkeland, S.E., Stepaniak, L. and Sbrhaug, T., 1985. Quantitative studies of heat-stable proteinase from Pseudomonas fluorescens P1 by the enzyme-linked immunosorbent assay. *Appl. Environ. Microbiol.* 49: 382– 387.

Bhat, M., Denny, J., Clark, D., Hofmann. J., Jain, S. and Lynch, M. 2007. *Escherichia coli* O157: H7 infection associated with drinking raw milk---- Washington and Oregon, November-December 2005. *J. Am. Med. Association.* 297: 1426-1428

Chye, F.Y., Abdullah, A. and Ayob, M. K. 2004. Bacteriological quality and safety of raw milk in Malaysia. *Food Microbiol.* 21: 535-541

Cousin, M.A., Jay, J.M. and Vasavada, P.C. 2001. Psychrotrophic microorganism.

Compendium of methods for the microbial examination of foods. (eds. Downes, F.P. and Ito, K.). Fourth edition. American Public Health Association, Washington D.C, pp. 159-164.

Cremonesi, P., Castiglioni, B., Malferrari, G., Biunno, I., Vimercati, C., Moroni, P., Morandi, S. and Luzzana, M. 2006. Technical note: Improved method for rapid DNA extraction of mastitic pathogens directly from milk. *J. Dairy Sci.* 89: 162-169

Daly, P., Collier, T. and Doyle, S. 2002. PCR-ELISA detection of *Escherichia coli* in milk. *Lett. Appl. Microbiol.* 34: 222-226

Deng, M.Y., Cliverb, D.O., Day S.P. and Fratamico, P.M. 1996. Enterotoxigenic *Escherichia coli* detected in foods by PCR and an enzyme-linked oligonucleotide probe. *Int. J. Food Microbiol.* 30 : 217-229

Desmarchelier, P.M., Bilge, S.S., Fegan, N., Mills, L., Vary, J.C.JR. and Tarr, P.I. 1998. A PCR specific for *Escherichia coli* O157 based on the *rfb* locus encoding O157 lipopolysaccharide. *J. Clin. Microbiol.* 36: 1801-1804

Devatkal, S., Mendiratta, S.K., Bhilengaonkar, K.N. and Purshottam. 2005. Application of polymerase chain reaction for evaluation of microbial quality of buffalo liver. *J. Vet. Pub. Hlth.* 3: 99-103

Di Pinto. A., Forte. V.T., Guastadisegni, M.C., Martino, C., Schena, F.P. and Tantillo. 2007. A comparison of DNA extraction methods for food analysis. *Food Control.* 18: 76-80

Dogan, B. and Boor, K.J. 2003. Genetic diversity and spoilage potentials among Pseudomonas spp. isolated from fluid milk products and dairy processing plants. *Appl. Environ. Microbiol* 69: 130-138

Eneroth, A., Christiansson, A., Brendehaug, J. and Molin, G. 1998. Critical contamination sites in the production line of pasteurized milk, with special reference to the psychrotrophic spoilage flora. *Int. Dairy J.* 8: 829-831

Eneroth, A., Ahrne, S. and Molin, G. 2000. Contamination of milk with gram-negative spoilage bacteria during filling of retail containers. *Int. J. Food Microbiol.* 57: 99–106

Fach, P. and Popoff, M.R. 1997. Detection of enterotoxigenic *Clostridium perfringens* in food and faecal samples with a duplex PCR and the slide latex agglutination test. *Appl. Environ. Microbiol.* 63: 4232-4236

Feng, P. 1995. *Escherichia coli* serotype O157:H7 novel vehicles of infection and emergence of phenotypic variants. *Emerging infectious diseases.* 1: 47-52

Fode-Vaughan, K.A., Maki, J.S., Benson, J.A. and Collins, M.L.P. 2003. Direct PCR detection of *Escherichia coli* O157:H7. *Lett. Appl. Microbiol.*37: 239-243

Forsman, P., Timisjarvi, A. T. and Alatossava, T. 1997. Identification of Staphylococcal and Streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. *Microbiology.* 143: 3491-3500

Furet, J.P., Quenee, P. and Tailliez, P. 2004. Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *Int. J. Food Microbiol.* 97: 197- 207

Gillespie, B. E. and Oliver, S. P. 2005. Simultaneous detection of mastitis pathogens *Staphylococcus aureus Streptococcus uberis* and *Streptococcus agalactiae* by multiplex polymerase chain reaction. *J. Dairy Sci.* 88: 3510-3518

Gini George. 2007. Microbial quality and safety of raw milk with reference to sources of contamination.M.V.Sc. thesis, Kerala Agricultural University, Thrissur, p.172

Gonzalez, I., Martin, R. Gracia, T., Merales, P., Sanz, B., and Hernandez, P. E. 1994. Rapid enumeration of *Escherichia coli* in oysters by a quantitative ELISA-PCR. *J. Appl. Microbiol.* 86: 231-236

Gonzalez, I., Gracia, T., Fernandez, A., Sanz, B., Hernandez, P. E. and Martin, R. 1999. Polyclonal antibodies against live cells of Pseudomonas fluorescens for the detection of psychrotropic bacteria in milk using a double antibody sandwitch enzyme-linked immunosorbent assay. *J. Dairy Sci. 77*: 3552-3557

Griffiths, M. W. and Philips, J. D. 1988. Modelling the relation between bacterial growth and storage temperature in pasteurized milk of varying hygienic quality. *J. Soc. Dairy Technol.* 41: 96-102

Grover, S. and Srinivasan, R. A. 1988. Isolation and characterization of *Pseudomonas aeruginosa* from milk and milk products. *Indian J. Dairy Sci.* 41: 326-329

Gupta, V. K., Verma, D. K., Rout, P. K., Singh, S. V. Vihan. V. S. 2006. Polymerase chain reaction (PCR) for detection of *Brucella melitensis* in goat milk. *Small Ruminant Research* 65: 79–84

Gutierrez, R., Gracia, T., Gonzalez, I., Sanz, B., Hernandez, P. E. and Martin, R. 1997. A quantitative PCR-ELISA for the rapid enumeration of bacteria in refrigerated raw milk. *J. Appl. Microbiol.* 83: 518-523

Gutierrez, R., Gracia, T., Gonzalez, I., Sanz, B., Hernandez, P. E. and Martin, R. 1998. Quantitative detection of meat spoilage bacteria by using the

polymerase chain reaction (PCR) and an enzyme linked immunosorbent assay. *Lett. Appl. Microbiol.* 26: 372-376

Henegariu, O., Heerema, N. A., Dllouhy, S. R., Vance, G. H. and Vogt, P. H. 1997. Multiplex PCR: Critical parameters and step by step protocol. *Biotechniques* 23: 504-511

Hill, W. E. 1996. The polymerase chain reaction: Applications for the detection of food borne pathogens. *Critical reviews in food science and nutrition.* 36:123-173

Holt, C. 1992. Structure and stability of bovine caseine micelles. *Advances in protein chemistry.* 43: 63-153

Hsu, H.Y., Chan, S.W., Sobell, D.I., Halbert, D.N. and Groody. E.P.1991. A colorimetric DNA extraction method for *Escherichia coli* in foods. *J. Food Prot.* 54:249-255

Hsu, S.C. and Tsen, H.Y. 2001. PCR primers designed from malic acid dehydrogenase gene and their use for detection of *Escherchia coli* in water and milk samples. *Int. J. Food. Microbiol.* 64:1-11

Indian Standards. 1980. SP: 18. *ISI Hand Book of Food Analysis.* Part I. Bureau of Indian Standards. Manak Bhavan, 9, Bahadur Shah Zafar Marg, New Delhi- 1. p. 85

Jaibi, K. 2006. Bacterial quality of raw milk at the co-operative society level with special reference to quality assurance programme. M.V.Sc. thesis, Kerala Agricultural University, Thrissur, p.178

Jayarao, B.M. and Henning, D.R. 2001. Prevalence of foodborne pathogens in bulk tank milk. *J. Dairy Sci.* 84:2157–2162

Jayarao, B.M. and Wang, L. 1999. A study on the prevalence of gram negative bacteria in bulk tank milk. *J. Dairy Sci.* 82:2620-2624

Jensen, M.A., Webster, J.A. and Straus, N.1993. Rapid identification of bacteria on the basis of polymerase chain reaction –amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* 59: 045-952

Jolly, D., Nanu, E. and Sunil, B. 2000. Evaluation of certain quality characteristics of raw market milk. *Smallholder Livestock Production System in Developing Countries.* p. 451-458

Kuzma, K., Malinowski, E., Lassa ,H. and Klossowska, A. 2003. Specific detection of *Staphylococcus aureus* by PCR in intramammary infection. *Bull. Vet. Inst. Pulawy* 47: 183-190

Lancette, G.A. and Bennett, R.W. 2001. *Staphylococcus aureus* and staphylococcal enterotoxins. *Compendium of methods for the microbiological examination of foods* (eds. Downes, F.P. and Ito, K.) Fourth edition. American public health association, Washington DC, pp. 387-403

*Lantz, P.G., Al-Soud, W.A., Knutsson, R., Hahn-Hagerdal, B. and Radstrom, P. 2000. biotechnical use of polymerase chain reaction for microbiological analysis of biological samples. *Biotechnol. Ann. Rev.* 5: 130

Latha, C. and Nanu, E. 1997. Bacteriological quality of pasteurized milk and its public health significance. Proceedings of the ninth Kerala Science Congress, Kerala, pp115-117

Lekha Chacko. 2006. Bacterial quality of milk at the point of production with special emphasis on the quality assurance programme. M.V.Sc. thesis, Kerala Agricultural University, Thrissur, p. 176

*Le Loir, Y., Baron, F. and Gautier, M. 2003. *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res.* 2: 63-76

Li, W. and Drake, M.A. 2001. Development of a quantitave competitive PCR assay for detection and quantification of *Escherichia coli* O157:H7 cells. *Appl. Environ. Microbiol.* 67: 3291-3294

Lindqvist, R. 1997. Preparation of PCR samples from food by a rapid and simple centrifugation technique evaluated by detection of *Escherichia coli* 0157:H7. *Int. J. Food Microbiol.* 37: 73-82

Lucore, L., Cullison, M.A. and Jaykus, L. 2000. Immobilization with metal hydroxides as means to concentrate food borne bacteria for detection by cultural and molecular methods. *Appl. Environ. Microbiol.* 66: 1769-1776

Lues, J.F.R., Venter, P., van der Westhuizen, H. 2003. Enumeration of potential microbiological hazards in milk from a marginal urban settlement in central South Africa. *Food Microbiol.* 20: 321–326

Mamani, Y., Quinto, E.J., Gandara, S.J. and Mora, M.T. 2003. Growth and survival of *Escherichia coli* O157 : H7 in different types milk stored at 4°C or 20°C. *J. Food Sci.* 68: 2558-2563

Manna, S.K., Brahmane, M.P., Das, R., Chandana, M. and Batabyal, S. 2006. Detection of *Escherichia coli* O157 in foods of animal origin by culture and multiplex polymerase chain reaction. *J. Food Sci Technol.* 43: 77-79

Marcos, J.Y., Soriana, A.C., Salazar, M.C. Moral, C.H., Ramos, S.S., Smeltzer, M.S. and Carrasco, G.N. 1999. Rapid identification and typing of *Staphylococcus aureus* by PCR restriction fragment length polymorphism analysis of the *aroA* gene *J. Clin. Microbiol* 37: 570-574

Martinez, G., Harel, J. and Gottschalk, M. 2001. Specific detection by PCR of *Streptococcus agalactiae* in milk. *The Canadian J. Vet. Res.* 65: 68-72

Martins, M.L., de Araujo, E.F., Mantovani, H.C., Moraes, C. ., Maria, C.D. and Vanetti, T. 2005. Detection of the *apr* gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. *Int. J. Food Microbiol.* 102: 203– 211

May, S.A., Mikolajcikm, E.M. and Richter, E.R. 1989. Conventional tube and microplate limulus amoebocyte lysate procedures for determination of gram negative bacteria in milk. *J. Dairy Sci.*72: 1137-1141

McKillip, J.L., Jaykus, L.A. and Drake, M.A. 2000. A comparison of methods for the detection of *Escherichia coli* O157:H7 from artificially contaminated dairy products using PCR. *J. Appl. Microbiol.* 89: 49-55

Meiri-Bendek, I., Lipkin, E., Friedmann, A., Leitner, G., Saran, A., Friedman, S. and Kashi, Y. 2002. A PCR-based method for the detection of *Streptococcus agalactiae* in milk. *J. Dairy Sci.* 85: 1717-1723

Meng, J. Zhao, S., Doyle, M.P., Mitchell, S.E. and Kresovich, S. 1996. Polymerase chain reaction for detecting *Escherichia coli* O157: H7. *Int. J. Food Microbiol.* 32: 103-113

Moon, G.S., Kim, W.J. and Shin, W.S. 2004. Optimization of rapid detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by PCR and application to field test. *J. Food Prot.* 67: 1634-1640.

Mortan, D.R. 2001. Aerobic plate count. *Compendium of methods for the microbial examination of foods.* (eds. Downes, F.P. and Ito, K.). Fourth edition. American Public Health Association, Washington DC, pp. 63-68

Muramatsu, Y., Maruyama., M., Yanase, T., Ueno, H. and Morita, C. 1996. Improved method for preparation of samples for the polymerase chain reaction for detection of *Coxiella burnetii* in milk using immunomagnetic separation *Vet. Microbiol.* 51: 179- 185

Murphy, M.A., Shariflou, M.R. and Moran, C. 2002. High quality genomic DNA extraction from large milk samples. *J. Dairy Res.* 69: 645-649

Nakano, S., Kobayashi, T., Funabiki, K., Matsumura, A., Yasuhira, N. and Yamada, T. 2004. PCR detection of Bacillus and Staphylococcus in various foods. *J. Food Prot.* 67: 1271-1277

Nakayama, A., Okoyama, A. Hashida, M., Yamamoto, Y., Takebe, H., Ohnaka, T. Tanaka, T. and Imai, S. 2006. Development of a routine detection system of staphylococcal enterotoxin genes. *J. Medical Microbiol.* 55: 273-277

Nanu, E., Latha, C., Sunil, B., Prejit, Thomas, M. and Menon, K.V. 2007. Quality assurance and public health safety of raw milk at the production point. *Am. J. Fd. Technol.* 2: 145-152

Naraveni, R and Jamil, K. 2005. Rapid detection of food borne pathogens by using molecular techniques. *J. Med. Microbiol.* 54: 51-54

Neeley, E.T., Phister, T.G. and Mills, D.A. 2005. Differential real time PCR assay for enumeration of lactic acid bacteria in wine. *Appl. Microbiol. Biotechnol.* 71: 8954-8957

Oberst, R.D., Hays, M.P., Bohra, L.K., Phebus, R.K., Yamashiro, C.T., Paszko-Kolva, C., Flood, S.J.A. and Gillespie, J.R. 1998. PCR – based DNA amplification and presumptive detection of *Escherichia coli* O157:H7 with an internal fluorogenic probe and the 5' nuclease (taqman) assay. *Appl. Environ. Microbiol.* 64 : 3389-3396

Oksuz, O., Arici, M. Kurultay, S. and Gumus, T. 2004. Incidence of *Escherichia coli* O157 in raw milk and white pickled cheese manufactured from raw milk in Turkey. *Food Control.* 15: 453–456

Padhye, N.V. and Doyle, M.P. 1991. Rapid Procedure for detecting enterohemorrhagic *Escherichia coli* 0157:H7 in food. *Appl. Environ. Microbiol.* 57: 2693-2698

*Panet, A. and Khorana, H.G. 1974. Studies on polynucleotides. The linkage of leoxyribonucleic templates to cellulose and its use in their replication. *J. 3iol. Chem.* 249: 5213-5221

Peles, F., Wagner, M., Varga, L., Hein, I., Rieck, P., Gutser, K., Kereszturi, P., Kardos, G., Turcsányi, I., Beri, B. and Szabo, A. 2007. Characterization of *Staphylococcus aureus* strains isolated from bovine milk in Hungary. *Int. J. Food Microbiol.* 118: 186–193

Phuektes, P., Mansell, P.D. and Browning, G.F. 2001. Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and Streptococcal causes of bovine mastitis. *J. Dairy Sci.*, 84: 1140-1148

Powell, H.A., Gooding, C.M., Garrett, S.D., Lund, B.M. and McKee, R.A. 1994. Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. *Lett. Appl. Microbiol.* 18: 59-61.

Prakash, M. Rajasekar K. and Karmegam N. 2007. Bacterial population of raw milk and their proteolytic and lipolytic activities. *Research Journal of Agriculture and Biological Sciences,* 3(6): 848-851

Prejit. 2005. Microbial quality assurance of milk in its production, processing and storage. M.V.Sc. thesis, Kerala Agricultural University, Thrissur, p.157

Rall, V.L.M., Vieira, F.P., Rall, R., Vieitis, R.L., Candeias, J.M.G., Cardoso, K.F.G., Araujo Jr. J. P., 2008. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. Available: http://www.ncbi.nlm.nih.gov/pubmed/18572331 (Accessed 17 May 2008)

Ramesh, A., Padmapriya, B.P., Chandrashekar, A. and Varadaraj, M.C. 2002. Application of a convenient DNA extraction method and multiplex PCR for direct detection of *Staphylococcus aureus* and *Yersinia enterocolitica* in milk samples. *Mol. Cell. Probes.* 16; 307-314

Rantsiou, K.A., Alessandria, V., Urso, R., Dolci, P. and Cocolin, L. 2008. Detection, quantification and vitality of *Listeria monocytogenes* in food as determined by quantitative PCR. *Int. J. Food Microbiol.* 121: 99–105

Rey, J., Sanchez, S., Blanco, J.E., de Mendoza, J.H., Garcıa. A., Gil, C., Tejero, N., Rubio, R. and Alonso, J.M. 2006. Prevalence, serotypes and virulence genes of Shiga toxin-producing *Escherichia coli* isolated from ovine and caprine

milk and other dairy products in Spain. *Int. J. Food Microbiol.* 107: 212 – 217

Riffon, R., Sayasith, K., Khalil, H., Dubreuil, P., Drolet, M. and Lagace, J. 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *J. Clin. Microbiol.* 39: 2584-2589

Romero, C. and Lopez-Goni, I. 1999. Improved method for purification of bacterial DNA from bovine milk for detection of Brucella spp. by PCR. *Appl. Environ. Microbiol.* 65: 3735-3737

Rossen, L., Norskov, P., Holmstrom, K. and Rasmussen.1992. Inhibition of PCR by components of food samples, microbilogical diagnostic assays and DNA extraction of solutions. *Int. J. Food Micobiol.* 17: 37-45

Sethulekshmi, C., Jacob, R.R., Nanu, E. and Sunil, B. 2003. Bacterial quality of toned pasteurized milk retailed in and around Thrissur. Poster abstracts of fifth international food convention, 5-8 December 2003, Mysore, India, *Abstract*: 57

Spilker, T., Coenye, T., Vandamme, P. and Lipuma, J.J. 2004. PCR based assay for differentiation of *Pseudomonas aeruginosa* from other Pseudomonas species recovered from cystic fibrosis patients *J. Clin. Microbiol.* 42: 2074-2079

Soomro, A.H., Arain, M.A., Khaskheli, M. and Bhutto, B. 2002. Isolation of *Escherichia Coli* from raw milk and milk products in relation to public health sold under market conditions at Tandojam. *Pak. J. Nutrition* 1: 151-152

Straub, J.A., Hertel, C. and Hamers, W.P. 1999. A 23S rDNA targeted polymerase chain reaction-based system for detection of staphylococcus meat starter cultures and dairy products. *J. Food Prot.* 62: 1150-1156

Sutherland, A.D., Limond, A.M., MacDonald, F. and Hirst, D. 1993. Evaluation of the incubated plate count test for pasteurized milk. *J. Soc. Dairy Technol.* 46: 107-113

Ternstrom, A., Lindberg, A.M. and Molin, G. 1993. Classification of the spoilage flora of raw and pasteurized bovine milk, with special reference to *Pseudomonas* and *Bacillus*. *J. Appl. Bacteriol.* 75: 25-34

Vaitilingom, M., Gendre, F. and Brignon, P. 1998. Direct detection of viable bacteria, molds and yeasts by reverse transcriptase PCR in contaminated milk samples after heat treatment. *Appl. Environ. Microbiol.* 64: 1157-1160

Venkitanarayana, K.S., Faustman, J.F., Khan, M.I., Hoagland, T.A. and Berry, B.W. 1997. Rapid estimation of spoilage bacterial load in aerobically stored meat by a quantitative polymerase chain reaction *J. Appl. Microbiol.* 82: 359-364

Wang, R.F., Cao, W.W. and Cerniglia, C.E. 1997. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. *J. Appl. Microbiol.* 83: 727 -736

Weidmann, M., Weilmeier, D., Dineen, S.S. Ralyea, R. and Boor, K.J. 2000. Molecular and phenotypic characterization of Pseudomonaas spp. isolated from milk. *Appl. Environ. Microbiol.* 66: 2085-2095

Wegmuller, B., Luthy, J. and Candrian, U. 1993. Direct polymerase chain reaction detection of *Campylobacter jejuni* and *Campylobacter coli* in raw milk and dairy products. *Appl. Environ. Microbiol.* 59: 2161-2165

Wilson, I.G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* 63: 3741-3751

Winters, D.K., Oleary, A.E. and Slavik, M.F. 1998. Polymerase chain reaction for rapid detection of *Campylobacter jejuni* in artificially contaminated foods. *Lett. Appl. Microbiol.* 27: 163-167

Wu, S.J. and Kado, C.I. 2004. Preparation of milk samples for PCR analysis using a rapid filtration technique. *J. Appl. Microbiol.* 91: 1342-1346

Yadava, R., Choudhary, S.P. and Narayan, K.G. 1985. Bacterial flora of market milk and its public health importance. *Indian J. Dairy Sci.* 38: 235-237

Yang, H., Qu, L., Wimbrow, A.N., Jiang, X. and Sun, Y. 2007. Rapid detection of *Listeria monocytogenes* by nanoparticle-based immunomagnetic separation and real-time PCR. *Int. J. Food Microbiol.* 118: 132–138

Yang, Y., Xudong, S., Yaowu,Y., Chunyu, K., Yingjun, L.,Weil, Z. and Ximyingz, Z. 2007. Detection of *Staphylococcus aureus* in dairy products by polymerase chain reaction assay. *Agricultural Sciences in China.* 6: 857-862

Yokoigawa. K., Inoue. K., Okubo. Y. and Kawai, H. 1999. Primers for amplifying an alanine racemase gene fragment to detect *E. coli* strains in foods. *J. Food Sci.* 64: 571-575

* originals not consulted

APPLICATION OF POLYMERASE CHAIN REACTION FOR RAPID EVALUATION OF HYGIENIC STATUS OF MILK

DEEPA MARY. J. J.

Abstract of the thesis submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

2008

Department of Veterinary Public Health
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

ABSTRACT

Rapid assessment of the bacterial load and detection of pathogens in milk is of public health significance. Molecular detection of pathogenic microorganisms is based on DNA amplification of the target pathogens. Therefore efficient extraction of DNA directly from milk is a major step.

DNA could be efficiently extracted directly from milk by a prior sample preparation so as to remove the fat and milk proteins. The phenol chloroform method of DNA extraction was modified to reduce the time require for the procedure. The use of lysozyme helped the release of DNA from lysed gram positive *Staphylococcus aureus.* The extracted DNA was used as template in PCR. PCR was carried out with already published primers. PCR was modified with the use of PCR buffer containing PCR facilitators (BSA and Tween 20) to overcome PCR inhibition. The standardized procedure was used to assess the bacterial load and to detect *Escherichia coli* and *S. aureus* directly from milk.

To assess the bacterial load dilutions of milk were made upto10^{-10}. DNA was extracted from each dilution with which PCR was carried out with primers specific for Pseudomonas. Aerobic Plate Count was also done for the same samples and compared with PCR. It could be concluded that the approximate APC of the milk sample by PCR is next lower dilution to the dilution giving the PCR amplification. The total time taken for the analysis was approximately five hours.

Extraction of DNA and PCR was done with primers for detection of *E. coli* from the same milk samples and compared with culture. Percentage of samples positive both by culture and PCR was 50 and negative by both methods were 30. Twenty percentage of the samples were positive by PCR and negative by culture.

Extraction of DNA and PCR was done with primers for detection of *S. aureus* from the same milk samples and compared with culture. Percentage of samples positive both by culture and PCR was 60 and negative by both methods were 20. Twenty percentage of the samples were negative by PCR and positive by culture. Hence, protocol developed for detection of *S. aureus* needs further refinement to take care of false negative results by PCR, probably due to the low number of organisms present in milk.