POLYMERASE CHAIN REACTION BASED SCREENING OF BOVINE MASTITIS MILK TO DETECT *Leptospira* AND *Mycoplasma*

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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

2007

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DECLARATION

I hereby declare that the thesis, entitled **"POLYMERASE CHAIN REACTION BASED SCREENING OF BOVINE MASTITIS MILK TO DETECT *Leptospira* AND *Mycoplasma.*"** 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.

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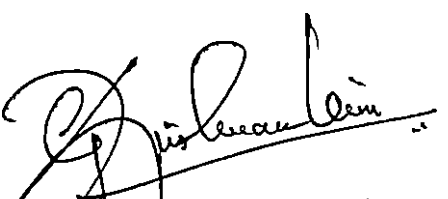

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ACKNOWLEDGEMENTS

I am at loss of words to express my heartfelt gratitude to ***Dr. G. Krishnan Nair,*** *Associate Professor and Head, Department of Veterinary Microbiology and the Chairman of my Advisory Committee for his valuable guidance ,ever willing help, , perpetual support, constant encouragement .Without his advice and strong support, the successful completion of this work would not have been possible.*

With profound gratitude and indebtedness, I would like to express my deep sense of obligation and gratefulness to ***Dr. M. Mini,*** *Associate Professor, Department of Veterinary Microbiology for offering her experience, never ending help, valuable advice, constant encouragement and untiring efforts throughout the course of study.*

I am greatly indebted to ***Dr. Koshy. John,*** *Assistant Professor (SS), Department of Veterinary Microbiology and a member of Advisory Commmittee for his help, guidance and support as a member of the advisory committee.*

With great respect I would like to express my sincere gratitude to ***Dr. Saseendranath,*** *Associate Professor and Head, Department of Veterinary Epidemiology and Preventive Medicine and a member of Advisory Committee for offering constant encouragement valuable suggestions during my studies.*

I was fortunate enough to have ***Dr. T.V. Aravindakshan,*** *Assistant Professor (SS), Centre for Advanced Studies in Animal, Genetics and Breeding , as a member of the Advisory Committee. I am deeply indebted for his relevant suggestions and timely encouragement .*

I am cordinally obliged to ***Sri.P.R.Chandrasekhar****, Instrumentation Engineer and Head ,Central Instrumentation lab for the timely help rendered during the course of my work.*

I gratefully acknowledge the help and support offered to me by ***Dr. Aravind Ghosh*** *during the course of my work.*

Let me place on record my deep gratitude for timely help and support rendered for me by ***Dr. Sandhya Achuthan****.*

The timely help, support and friendship extended by ***Drs. Siji, Raseena, Sumena, Raji, Manjusha, Seena , Soumya, and Preethymol*** *are greatly acknjowledged.*

I sincere acknowledge the valuable help rendered to me by ***Dr. Rajagopal and Dr.Sunil****. I am warmly remembered and acknowledge my colleagues* ***Drs. Ambily, Ranjini and Jesto.***

With great pleasure, I am thanking my respectful seniors ***Drs. Dhannia, Dipu, Sunitha Karunakaran and Aparna****. I warmly remember and acknowledge my juniors* ***Drs. Anupama and Indu.***

I would like to express my sincere thanks to ***Dr. Ajmal and Dr. Anju*** *who helped me in collecting articles from Madras Veterinary College.*

I sincerely thanks the generous help rendered by ***Binoy, Biju, Nishanth and Vivek.***

I am greatful to the Non-Teaching Staff of the Department of Microbiology, ***Mrs. M.V. Kumari, Mrs. Leela , Mrs. Kamala and Mr. Balu*** *for their whole hearted help and immense effection.*

I am greatful to ***Dr.E. Nanu,*** *Dean, College of Veterinary and Animal Sciences, Mannuthy for providing the facilities needed for carrying out this work. I am indebted to* ***Kerala Agricultural University*** *for awarding me the fellowship needed for postgraduate study.*

I am in short of words to express my deep sense of love and gratitude to my beloved ***Achan, Amma and Chetan.***

Above all, I bow my head before ***God*** *for all the blessings showered on me and leading me to the successful completion of this work.*

Jibi.M. G.

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INTRODUCTION

India is basically an agricultural country, possessing a cattle population of 220 million white cattle and 94 million buffaloe which is about 16.3 per cent and 56.6 per cent of the total world population, respectively. After stagnating between 1950 and 1970 at around 17-21 million tonnes, India had made a remarkable progress in milk production through white revolution (Mudgal *et al.,* 2003). In 2001, our milk production had reached 81 million tonnes and India became world leader in milk production. The Indian share of cow milk production to world is about 69.46 per cent. India's dairy generates an annual business worth Rs.550 billion and was expected to touch Rs.880 billion by the turn of the century (Bansil *et al.*, 2006)

Dairy sector constitute a lion's share in the value of output from livestock sector. Since time immemorial, milk and milk products have been accepted as a must in the dietery of people. In good olden days the population was small and life was simple. With the growth of population and change in pattern of life there was a rapid increase in demand for milk and milk products. The enormous demand on high and sustained milk production has led the animals to a state of production stress, thereby resulting in the emergence of production diseases. Mastitis is considered as one of the major economic diseases in dairy sector because it attributes more than 25 per cent of all the disease related economic losses in dairy industry. It is the single most common disease syndrome in adult dairy cows, accounting for 38 per cent of all morbidity. It has been reported that on an annual basis three of every ten dairy cows have clinically apparent inflammation of the mammary gland. Of the affected cattle, seven per cent are culled and one per cent die as a consequence of the disease (Bradford *et al.*, 1982). According to Raubertas and Shook (1982) each doubling of average lactation somatic cell count (SCC) above 50,000 is associated with 400 lb production loss in mature cows and 200 lb production loss in first lactation cows.

Mastitis is defined as inflammation of udder caused by microbes/trauma of udder. Organisms associated with mastitis can be classified into two: contagious and environmental pathogens. Organisms which are seen in cow's environment gain entry through the streak canal, multiply in the udder and produces disease. The National Mastitis Council recommends a time proven five point control programme to solve this disease like milking machine maintenance, teat dipping, early treatment, dry cow therapy and culturing of milk from cows with chronic mastitis. All these control strategies adopted by the farmer have found success in decreasing the prevalence of traditional pathogens such as *Streptococcus agalactia* and *Staphylococcus* .

Eventhough traditional pathogens are under control, mastitis cases continues from which we could not isolate any of these organisms. Hence it is assumed that there are some unrecognised fastidious organisms associated with mastitis which can not be easily diagnosed by routine laboratory examinations. Epidemiological studies revealed that *Leptospira* and *Mycoplasma* are two of such fastidious organisms associated with mastitis and the incidence of these organisms has increased in the past few years.

Leptospirosis is not a primary disease of udder. The presence of leptospires in the udder seemed to be associated with the bacteraemic phase of infection (Ellis *et al.*, 1976) which will multiply in the udder and produce disease. Infection in cattle has been classified into two major groups: Incidental infection produced by the strain which is adapted to cattle like *Leptospira hardjo* and accidental infection produced by strains not adapted to cattle like *L.pomona. Leptospira pomona is* thought to be largely derived from wild life reservoirs and produce severe disease only on accidental exposure to this strain. Both serovars may cause systemic illness, abortion, mastitis and neonatal death (Ellis *et al.*, 1982). Cattle which act as the carrier host for leptospiral organism will excrete the organism for long time through urine. Organism can survive in alkaline soil

for a long period, and act as source of infection to other animals and human beings.

The clinical signs exhibited by the animal are not distinctive but are only suggestive of leptospiral mastitis. Milk of the affected animal will be thick, almost like colostrum, and occasionally blood tinged. The udders are flaccid and all the four quarters are equally affected. Frequent pyrexia and decrease in rumen movements are also noticed. Agalactia last for two to ten days and then production returns to normal except for cows in late lactation. Most chronic sequelae in pregnant animals are abortion, still birth and prematurely born weak calves. Most abortions occur during the last trimester of pregnancy. Urine drinking is a noticeable feature in the herds and will provide a mean for transmission of leptospirosis. *Leptospira hardjo* infection is usually characterized by mild clinical signs. It is found to be the most important serovar in cattle since it is maintained by chronic shedders causing new infections every year.

Prevalence of *Mycoplasma* mastitis appears to be increasing in many locations throughout the world. *Mycoplasma* is considered to be a contagious mastitis pathogen. Most of the organisms will colonize as commensals in the respiratory tract of calves and cows and these have a potential to become pathogen. But it is not clear what factors might induce the change from an asymptomatic to a diseased state. Internal transmission of *Mycoplasma* takes place via haematogenous and lymphatic routes. The bovine mammary gland is probably more susceptible to *Mycoplasma* infections than any other site because it doesn't normally posses any resident *Mycoplasma*. Despite clear evidence of its pathogenic potential, detailed knowledge about the major mechanism of pathogenesis is still scarce. Cytoadhesion is assumed to be crucial step in the initial phase of infection (Thomas *et al.,* 2003). Organisms have the ability to create at least a temporary defect in the full development of both humeral and cellular immunity. Given this delay, the organism will readily populate in the mammary tissue and produce clinical mastitis.

Gonzalez and Wilson (2003) listed eleven mycoplasmas that have been associated with mastitis, of which *M.bovis* was the most prevalent one. Clinical signs produced by *Mycoplasma* are characteristic, enabling to distinguish from other mastitis pathogens. Jasper (1977) opined that *Mycoplasma* should be suspected whenever there is, an increase in purulent mastitis cases that resist treatment: however, cows continue to eat and have very little evidence of illness, except an early transitory fever which usually escapes detection. *Mycoplasma* mastitis typically involve more than one quarter (often all quarters). Abnormal secretions with tannish or brownish discolourations, sandy or flaky sediments in watery or serous fluids are also noticed.

Leptospirosis, which is considered as occupational hazard to dairy workers, reveals the relevance of early diagnosis of this dreadful zooanthroponosis. Similarly in the case of mycoplasmosis it is crucial to have rapid and reliable diagnostic methods allowing detection of the agent at an early stage so that effective control measures can be introduced in time. Isolation of organisms is the most confirmatory method of diagnosis in both leptospirosis and mycoplasmosis. Requirement of enriched media and the slow growth characteristics of these fastidious organisms make the isolation technique tedious and time consuming. This delays the clinician to make a quick and prompt diagnosis. Moreover, the sensitivity of culture based methods depends on aseptic collection of sample and the survival of organisms under the condition of transit.

Serodiagnosis also does not provide a quick and reliable diagnosis because antibody titers are reached only by 10-14 days after the onset of clinical symptoms in mycoplasmal infection whereas anamnastic responses complicate interpretation of serological diagnosis in leptospirosis. It does not provide reliable information on the carrier or shedding state. Consequently, the pathogen cannot be detected during the incubation period.

Some of these drawbacks have been overcome by the introduction of molecular biology methods such as polymerase chain reaction (PCR). They are rapid and reduce the assay time from weeks to day or hours and can detect viable as well as non viable organisms. Thus PCR techniques hold much promise in the detection of fastidious organisms like *Leptospira* and Mycoplasma from mastitic milk.

Hence the present study has been undertaken with the following objectives

1. Detection of *Mycoplasma* and *Leptospira* in mastitis milk by PCR
2. Isolation of *Leptospira* and *Mycoplasma* from mastitis milk.

Review of Literature

2. REVIEW OF LITERATURE

2.1 LEPTOSPIROSIS - HISTORY

Leptospirosis was first reported in Germany by Weil in the year 1886.The disease was manifested as severe icterus and renal failure so called icteric leptospirosis. The term Weil's disease was first used by Goldsmidt in 1887 to denote the condition described by Weil. The term *Leptospira* was proposed by Nougchi (1918). Leptospirosis is a general term used to describe all the disease caused by genus *Leptospira* in all warm blooded animals. The genus *Leptospira* belongs to the family *Leptospiraceae,* order *Spirochaetales*. The classification of this organism is complex. Before 1989, all of pathogenic isolates belonged to species *Leptospira interrogans*, which contained more than 200 serovars in 23 serogroups. More recently, the genus *Leptospira* has been reclassified into 16 or more species. Pathogenic serovars are now found in the species *Leptospira interrogans*, *L.noguchii, L.santarosai, L.meyeri, L.borgpetersenii, Lkirschneri, L.weilii, L.inadai, L.fainei* and *L.alexanderi.* The new classification system can be confusing because both pathogenic and non pathogenic serovars and serogroups occur in the same species and a single serovar or serogroup can occur within multiple species. Serovars associated with disease in cattle include *hardjo, pomona, grippotyphosa, canicola and icterohaemorrhagiae* (Yasuda *et al.*, 1987: Perolat *et al.*, 1990: Ramadass *et al.*, 1992).

2.2 PREVALENCE

Hoare *et al.* (1972) found an outbreak of mastitis and abortion associated with *Leptospira hardjo* infection in cattle in New South Wales.

Leptospira interrogans serovar *hardjo* was recognized as a cause of distinctive acute agalactia in dairy cattle and as a major factor in bovine abortion in the United Kingdom. In Newzaeland, the strains Hardjoprajitno was isolated from clinical cases, and Hardjobovis was less pathogenic. This could be due to

the difference in distribution of strains in these countries and also difference in cattle management practices (Ellis *et al.*, 1988).

Bovine leptospirosis, a wide spread condition in Australia was associated with hemolytic disease, mastitis and reproductive loss in cows. The serovars commonly isolated from cattle in Australia were *pomona, hardjo* and *australis* (McClintock *et al.*, 1993).

Natarajaseenivasan and Ratnam (1997) found out sero-prevalence of leptospirosis in an organized farm in Madras city where calf mortality and repeat breeding were high, accompanied with drop in milk production. Pomona was predominant (34.5 per cent), followed by Sejroe (17.2 per cent), Autumnalis (13.8 per cent), Australis (8.6 per cent) and Tarassovi (3.4 per cent).

Leptospirosis caused by members of *Leptospira interrogans*, has emerged as an important zoonotic problem in India (Senthilkumar *et al.,* 1997).

Biswal *et al.* (2000) found that sero-prevalence of leptospirosis in cattle ranged from sixty to hundred per cent in Orissa state. Animals exhibited high titers to Javanica (26.9 per ent), Australis (15.2 per cent), Autumnalis (12.8 per cent), Pomona (8.6 per cent) and Icterohaemorrhagiae (5.1 per cent).

Black *et al.* (2001) conducted a study to find out prevalence of antibodies to *Leptospira* serovars in beef cattle in Central Queensland. Microscopic agglutination test (MAT) revealed that the major serovars were *hardjo* (15.8 per cent), *tarassovi* (13.9 per cent), *pomona* (4 per cent) and *szwajizak* (2 per cent). Antibodies to all other serovars were rarely detected.

The prevalence rate of leptospirosis as detected by Passive haemagglutination test (PHA) was 50.41 per cent in dogs, 23 per cent in cattle, 19.04 per cent in pigs, 26.19 per cent in rodents and 42.85 per cent in human beings in Thrissur, Kerala (Soman, 2004).

In a study conducted in MOSC Medical College, Kolenchery, Kerala, Autumnalis was found to be the most predominating serogroup during the period January 2000 to June 2002, followed by Pyrogenes, Grippotyphosa, Bataviae, Australis, Canicola, Icterohaemorrhagiae, Hebdomadis and Lousiana (Sugathan and Varghese, 2005).

2.3 DISEASE IN ANIMALS

2.3.1 Disease In Cattle

Ellis *et al.* (1976) could successfully isolate leptospires belonging to the Hebdomadis serogroup from milk and blood of cows with clinical mastitis. Affected cow appeared with all signs of flaccid mastitis. Milk and blood collected from affected animals were inoculated into hamster immediately. The samples were also used for isolation of leptospires four hours after collection. Leptospires were isolated from milk and blood by hamster inoculation and also by direct culture. Isolation was also made by direct inoculation of milk into Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium. The primary isolates grew only in modified EMJH medium. Organism was isolated from the milk of three out of five cows and the blood of two of those cows.

Ellis *et al.* (1982) opined that most of the cattle that had recently aborted due to leptospirosis had a high serum antibody titer. The serogroups that had been implicated in this condition were Icterohaemorrhagiae, Hebdomadis and Canicola.

Thiermann (1984) reported clinical signs like pyrexia, hemoglobinuria, jaundice, decreased milk production and red coloration of milk in adult cattle infected with leptospirosis.

Ellis *et al.* (1985) found that majority of *Leptospira* abortion occured from sixth month onwards and cows which aborted had not previously exhibited

overt signs of agalactia. There was an association between leptospiral infection and retention of fetal membranes.

Ellis *et al.* (1988) isolated *Leptospira interrogans* serovar *hardjo* from cattle in an abattoir, milk of agalactic cows and from aborted bovine fetuses.

Pereira (1991) found that serovar *hardjo* and related serovar within Sejroe were the most prevalent leptospires affecting the cattle in Portugal followed by *bratislava, canicola* and *pomona.* The high frequency of *hardjo* seroreactors among apparently normal cattle and their wide spread dispersion supported that *hardjo* was adapted to cattle and cattle acted as maintenance host for it.

McClintock *et al.* (1993) detected the age specific sero-prevalence in cattle affected with *hardjo.* Sero prevalence peaked once the heifers calved and joined the milking herd. Sero-prevalence in heifers had risen in the order of, before joining the herd (zero percent), and after joining the herd (21.7 per cent), cows in first lactation (53.1 per cent), and cows in second lactation (44 per cent).

Acute clinical signs occurred more often in leptospirosis in cows. Cattle might develop high fever (104-107^{0}C), depression, loss of appetite, decreased milk production and weakness. Hemoglobinuria, icterus and bloody milk were also seen (Hudson, 1996).

2.3.2 Disease In Sheep And Goat

Hathaway *et al.* (1982) found that leptospires of the Hebdomadis serogroup in sheep in many parts of England and Wales. Serovar *hardjo* was responsible for Hedbdomadis serogroup titers in sheep, as the organism was endemic in cattle in England and Wales.

Leptospirosis in sheep and goat occurred with less frequency than in cattle and swine. The signs reported were similar to those in cattle including high

fever, depression, and loss of appetite, decreased milk production and weakness. Signs like hemoglobinuria, anemia, icterus and bloody milk were also seen (Hudson, 1996).

2.3.3 Disease in Dogs

Dickeson and Love (1993) conducted epidemiological study to determine the prevalence of leptospiral infections in dogs and suggested that most prevalent serovar was *Leptospira interrorgans* serovar *canicola*.

The acute forms of leptospirosis in dogs caused elevated temperature, vomiting, and nephritis. In severe cases, jaundice and death might occur. The signs of central nervous system involvement could occur with or without other clinical signs and organism might be present in the brain tissue for extended period. Chronic leptospirosis was associated with chronic tissue degeneration. Shedding of leptospires in urine might continue for over a year (Hudson 1996).

2.3.4 Disease in Swine

Chronic leptospirosis is the commonest form of disease in pigs and is characterized by abortions and high incidence of stillbirths (Radostits *et al.*, 2000).

Immunofluorescence proved that *Leptospira* infection was common among fattening pigs in the Mekong delta. The *Leptospira interrogan* serovar *bratislava* was isolated from the kidneys of pigs. There was no association among presence of leptospires, multifocal interstitial nephritis and white spots in the kidney (Boqvist *et al.* 2003).

2.3.5 Disease In Humans

Mackintosh *et al.* (1981) carried out a case control study to investigate correlation between titers to *Leptospira* serovar in farm workers and those in

cattle in their herds. Twenty five milkers at high risk had titers of 1:96 or greater. Twenty seven were case controls and had no detectable agglutinin titers at minimum serum dilution of I: 24.

Biswal *et al.* (2000) found that the farm workers in Orissa exhibited sero-prevalence of 50 per cent for Icterohaemorrhagiae.

Boqvist et *al.* (2003) considered leptospirosis as an occupational zoonosis causing a health hazard for abattoir workers, meat inspectors and farmers.

2.4 RESERVOIR

Cattle were generally accepted as reservoir host for *hardjo* (Ellis *et al.*, 1981).

Gangadhar *et al.* (2000) attempted isolation of *Leptospira* from the kidneys of the *Rattus rattus*, *Wroughtoni hinton, Rattus rattus rufescens*, *Bandicota bengalensis* and *Bandicota indica.* In total, 296 *spirochetes* were isolated from 1,348 Kidney cultures, of which 23 isolates were identified as *L. inadai* based on serology and PCR analysis. The preponderance of *L. inadai* in four different species of rodents suggested that these animals could be the natural reservoir hosts of *L. inadai.*

Machang'u *et al.* (2004) found that any mammal could be infected with one or more *Leptospira* serovar. However, rodents were the most commonly affected animals, which were also the major natural reservoirs of *Leptospira.* In the tropics, peridomestic and field rodents such as *Rattus rattus, Mastomys natalensis* and *Cricetomys gambianus* were amongst the primary reservoirs of leptospires

2.5 MODE OF TRANSMISSION

Ellis *et al.* (1986) first isolated serovar *hardjo* from the genital tract and kidney of naturally infected bull. This finding supported the suggestion that venereal transmission of serovar *hardjo* could occur in cattle. It also provided further evidence that in host adapted leptospiral infections, the genital tract, as well as kidney might be an important sites for localization and persistence of organism in the maintenance host.

Heinemann (1999) suggested that bovine leptospirosis could be transmitted by artificial insemination since *Leptospira* could survive in the freezing temperature used to store semen.

2.6 CLINICAL SIGNS IN MASTITIS

California mastitis test (CMT) led to realization that some cows were sub clinically affected with *L.hardjo*. The milk production drop in these cows was not great, but the milk had an elevated cell count for about five to seven days (Davidson, 1971).

Hoare *et al.* (1972) reported that all four quarters were always affected, and there were no signs of swelling or inflammation. The milk sometimes contained yellow slimy clots, which often became quite hard and rubbery in later stages. A useful distinguishing feature even early in the course of the disease was the intensely positive CMT reaction in all four quarters.

Elise *et al.* (1976) found that milk of affected animal appeared thickened almost like colostrum and was occasionally blood tinged. The udders were flaccid and all four quarters were equally effected. Frequent pyrexia and decrease in rumen movements were also noticed. The illness in individual cows lasted for 14 days after which milk yields gradually returned to normal over a two weeks period. Urine drinking was a noticeable feature in this herd and would

provide a means of transmission. The presence of leptospires in the udder seemed to be associated with bacteraemic phase of infection.

Thiermann (1984) observed decrease in milk production in lactating cows. Mammary secretion was characterized by clots, pink or red discoloration. *Leptospira hardjo* infections were usually characterized by mild clinical signs: in case of agalactia, all quarters of the udder were affected and udder was soft and flabby. Agalactia lasted for two to ten days and production returned to normal except for cows in late lactation. The most chronic sequelae in pregnant animals were abortion, stillbirth, and prematurely born weak calves. Most abortion occurred during the last trimester of pregnancy. *Hardjo* was found to be the most important serovar in cattle since it was maintained by chronic shedders causing new infections every year.

2.7 DIAGNOSTIC METHODS

2.7.1 Microscopic Demonstration

2.7.1.1 *Dark Field Microscopy (DFM)*

Doherty (1966) examined urine by DFM and inferred that this method was superior to guinea pig inoculation technique.

Thiermann (1982) found that 1.25×10^{4} cells per ml of urine was required to observe a single organism by DFM.

Chandrsekaran and Pankajalakshmi (1997) demonstrated leptospires with DFM, by differential centrifugation of blood and concluded that DFM was useful in the early diagnosis of leptospirosis.

Sehgal *et al.* (2001) opined DFM as an ideal technique for demonstration of leptospires in culture, but was often used in demonstrating leptospires in clinical specimen, especially blood and urine, with less than satisfactory results.

2.7.1.2 *Staining*

Stimson (1907) demonstrated finally coiled organism with hooked ends in the renal tubules of a man believed to have died of yellow fever, by Levaditi's silver impregnation method and this was the first report of demonstration of leptospires in tissues.

Pargaonker (1957) observed *Leptospira* in the impression smears of kidneys and urine from rodents when stained with the silver method of Fontana and the microtome sections of kidneys with Levaditi's techniques.

2.7.1.3 *Immunoperoxidase*

Terpstra *et al.* (1983) demonstrated leptospires in blood and urine by immunoperoxidase staining method.

Ellis *et al.* (1983) used immunoperoxidase staining procedure for the detection of leptospires in tissues using antiserum to specific leptospiral serovars by which the presence and distribution of specific serovar in the tissue could be determined. . This technique was useful for the demonstration of leptospires in sections of formalin fixed paraffin embedded kidney tissue and found good correlation with cultural results.

Scanziani *et al.* (1991) made a comparison between specific immunoperoxidase staining and bacteriological culture in the diagnosis of renal leptospirosis. Compared with culture, the sensitivity of the immunoperoxidase procedure was 78 per cent and its specificity 100 per cent; the predictive value of a positive result was 100 per cent and of a negative result was 80 per cent.

2.7.2 Culture and Isolation of leptospires

Jayaprakasan *et al.* (1976) compared the growth and survivals of ten leptospiral serotypes belonging to different serogroups in media containing

bovine serum or extract of *Mycobacterium phlei* as enrichment with those in media containing rabbit serum. Bovine serum enrichment in the different media could not promote better growth of various serotypes when compared to the media containing rabbit serum, but all the serotypes showed good growth and remained viable for relatively long period with either enrichment. The survival of high percentage of all the serotypes in the Fletcher's medium with bovine serum enrichment during 43 days of observation very well substantiated the fact that these serotypes could be maintained in this medium for a long period of time.

Ellis *et al.* (1976) could successfully isolate leptospires belonging to the Hebdomadis serogroup from milk and blood of cows with clinical mastitis. He had used four media for isolation studies and found that EMJH was the only media that could support the growth of leptospires.

Tripathy *et al.* (1980) reported that serovars *hardjo* and *szwajizak,* produced colonial growth in bovine albumin polysorbate 80 (BAP 80) solid medium which had been supplemented either with sodium pyruvate or with five per cent rabbit serum. These results indicated that addition of either rabbit serum or sodium pyruvate would aid in promoting growth of some fastidious leptospires. Growth did not occur with any of these serovars in the unsupplemented BAP 80 medium.

Adler *et al.* (1986) developed Standard Albumin-Tween 80 medium (EMJH) for growth of leptospires and this was modified by addition of six antibiotics to produce a superior, selective medium for primary isolation of leptospires of serovars *hardjo* and *pomona* of *Leptospira interrogans* from clinical materials. But it was not possible to devise an antibiotic mixture which inhibited *Pseudomonas* while allowing the growth of leptospires.

Ellis *et al.* (1988) isolated the *Leptospira interrogans* serovar *hardjo* from cattle at an abattoir, milk of agalactic cows and from aborted bovine fetuses.

Pereira (1991) isolated serovar *hardjo* type *Hardjobovis* from the kidney sample of one year old apparently normal cattle. The isolates were recovered on primary isolation in semisolid EMJH medium.

McClintock *et al.* (1993) isolated serovar *pomona* and *zanoni* from dairy herd in north Queensland. Midstream urine was collected from second voiding after administration of frusemid and urine was immediately added to semi solid EMJH medium containing two per cent inactivated rabbit serum and 0.1 mg/ml 5-fluorouracil.

Shenberg (1997) described a chemically defined medium containing inorganic salts, vitamins, aminoacids, purines, pyrimidines and a fatty acid. This medium supported good growth of all *Leptospira.* All strains tested utilized ammonium chloride as a sole nitrogen source. Iron salt was essential for the growth of leptospires which was obtained from inorganic ferrous compounds, hemglobin, or hemin. In this media rapid growth was obtained from relatively small inoculum and cell crops of most strains tested were similar to those grown on serum containing media. The medium was also successfully used for the isolation of leptospires from infected tissues.

Natarajaseenivasan and Ratnam (1997) isolated serogroup Javanica from one field rat out of the attempts made in 34 field rats, urine samples of 30 normal cattle and 10 healthy human beings. Semisolid EMJH medium enriched with two per cent rabbit serum containing 100µg per ml of 5-fluorouracil as selective agent was used for the isolation.

Senthilkumar *et al.* (2001) opined that culture of *Leptospira* from the body fluids was the most demonstrative test but this technique was laborious and might take up to two months to get a result.

One hundred and twenty five samples collected from different sources like human, dogs, bovine and rodents were used for isolation of leptospires. Leptospires were isolated from three rodents only (Elaiyaraja, 2003).

2.7.3 Serological Diagnosis

Shortly after the onset of clinical signs, no sera had titers to *L. pomona* in cattle whereas rising titers to *L. hardjo* were observed. The titers usually rose rapidly and were often of the order of 1/3000 five to seven days after the onset of clinical signs. In a majority of cases the titers then fell rapidly to a level of 1/1000 or 1/300, but in some cases a higher titer persisted for extended periods. In cattle that aborted following a clinical episode of leptospiral mastitis, it was frequently observed that the titer was falling at the time of abortion (Hoare *et al.*, 1972).

Adler *et al.* (1981) compared Enzyme-linked immunosorbent assay (ELISA) with the standard microscopic agglutination test (MAT) as a method for detecting antibodies against *Leptospira interrogans* serovar *hardjo* in sheep.The two tests appeared to measure different antigen antibody system. They found that ELISA was a useful test for screening large numbers of serum for antibodies to *L.interrogans* serovar *hardjo*.

Cousins *et al.* (1985) found that IgM antibody levels increased in the first week, but became negative within three to five weeks in most animals, thus IgM ELISA appeared to be a suitable method of detecting recent exposure of leptospires in cattle. Enzyme-linked immunosorbent assay (ELISA) had a number of advantages over MAT. It used killed antigen, results could be read objectively rather than subjectively and it could measure different immunoglobulin classes without prior fractionation of sera.

Zuerner *et al.* (1988) observed that cattle infected with *Hardjobovis* might fail to produce detectable antibodies. An accurate diagnosis of infection

with Hardjobovis required direct demonstration of *Leptospira interrogans* in tissue.

Woodward (1997) carried out *Leptospira hardjo* sero diagnosis and made a comparison of MAT, ELISA and immunocomb. The percentage of positive results obtained by the immunocomb (31.5 per cent) and ELISA (31.3 per cent) both exceeded significantly ($P< 0.004$) the 29.3 per cent obtained by the MAT. However, comparison between the immunocomb and ELISA tests based on results diagnosed as positive and negative by MAT, showed that the ELISA was significantly more sensitive (88.4 per cent compared with 84.6 per cent, $P=0.010$) and also most specific (92.4 per cent compared with 90.6 per cent, $P=0.007$) than the immunocomb.

Ramadass *et al.* (1999) studied the efficacy of latex agglutination test (LAT) for rapid detection leptospiral antibodies. The efficacy of LAT was compared with the plate ELISA and percentage of positivities was 84.8 and 85.9 respectively. Even though the ELISA test was slightly more sensitive than LAT, the rapidity, simplicity and economics of the LAT were found to fulfill the requirements of a screening test for leptospiral antibodies.

Truccolo (2001) used an ELISA microtiter plate hybridization method for the quantitative determination of *Leptospira* species in biological samples after PCR. The biotin labeled amplified product (331 bp from the *rrs* gene) was hybridized with a complimentary capture probe covalently linked on to aminated poly styrene wells, and detected using a colorimetric reaction. The mean detection limit was 50 copies per µl, and density of 10^4 leptospires per ml of blood was a critical threshold for the vital prognosis of the patients.

2.7.4 Molecular Methods

2.7.4.1 *Polymerase Chain Reaction*

Zuerner *et al.* (1988) developed DNA hybridization probe to detect *Leptospira interrorgans* servovar *hardjo* type Hardjobovis in biological samples. The probe for this hybridization consisted of genomic DNA labeled by nick translation with radiolabeled or biotinylated nucleotides. This probe distinguished Hardjobovis from other pathogenic leptospires which commonly infected domestic animals.

Van Eys *et al.* (1989) first developed PCR for the detection of leptospires in urine samples of infected cattle. Urine samples were investigated using PCR assay, culture and isolation, dot and quick blot hybridization. This comparative study suggested that amplification by PCR might be a valuable method for the detection of leptospires in cattle urine. Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets, most frequently 16S or 23S genes and repetitive elements, while others have been constructed from genomic libraries.

Boom *et al.* (1990) developed a simple, rapid, and reliable protocol for the small scale purification of DNA and RNA from human serum and urine. This method was based on the lysing and nuclease inactivating properties of the chaotropic agent guanidine thiocynate together with the nucleic acid binding properties of silica particles in the presence of this agent. By using size fractionated silica particles, nucleic acid could be purified from twelve different specimens in less than one hour. The purified DNA was a good substrate for restriction enzyme analysis (REA) and DNA ligase and was recovered with high yields from the picogram to microgram levels.

The primers described by Merien *et al.* (1992) amplified a 331bp fragment of both pathogenic and non pathogenic leptospires, which in the unlikely event of contamination of specimen with non pathogenic leptospires might produce a false positive result. Meanwhile G1 and G2 Primers described by Gravekamp *et al.* (1993) did not amplify *L.kirschneri*, necessitating the use of two primer pairs for the detection of all pathogenic serovars.

Ralph *et al.* (1993) used Mapped Restriction Sight Polymorphism (MRSP) of PCR products from portions of *rrs* (16S rRNA gene) and *rrl* (23S rRNA gene) and genomic finger printing with Arbitrarily Primed PCR (AP-PCR) to categorise *Leptospira* species. The result of MRSP and AP-PCR methods confirmed high level of divergence among the recognized species of *Leptospira.*

Zuerner *et al.* (1993) had used IS 1553 probes to detect genetic difference among Hardjobovis isolates since many IS 1553 copies were distributed around the genome of Hardjobovis. By using these probes, 10 genetic groups of *L.borgpetersenii* serovar *hardjo* type Hardjobovis were identified.

Bal *et al.* (1994) reported that PCR analysis of urine could be valuable before sero conversion, in the first week of illness. PCR analysis of urine revealed persistent infection in cattle, hamsters, human as a result of localization of organisms in kidneys after antibiotic treatment.

Damas *et al.* (1994) said that Low- Stringency PCR (LS –PCR) differed from AP- PCR in that the choice of primer was no longer arbitrary, although conditions of the reactions remained the same. When specific primers were used under low stringency conditions the result was not only the amplification of the specific DNA sequence defined by the primers, but also production of a set of low stringency products as those that were produced by AP- PCR.

Perolat *et al.* (1994) characterized *Leptospira* serovar *hardjo* isolate of the Hardjoprajinitno and Hardjobovis genotype by AP-PCR fingerprinting and the

study of MRSP in *rrs* and *rrl* genes. Arbitrarily primed PCR (AP-PCR) fingerprint gave considerable evidence of genomic heterogenisity at the strain level among the Hardjobovis group. Conversely, the hardjoprajitno group was homogenous. Mapped Restriction Sight Polymorphism (MRSP) profiles in ribosomal genes indicated that Hardjoprajitno and Hardjobovis isolate belonged to *Leptospira interrogans* MRSP group B and *Leptospira borgopeteresenii* group C respectively. Arbitrarily primed PCR (AP-PCR) and determination of MRSPs in ribosomal genes proved to be quick and reliable methods for typing *Leptospira* strains and for studying intra specific population structures.

Brown *et al.* (1995) demonstrated that both PCR and culture were more often positive for sera than for urine samples and it could detect leptospires in sera even before the development of antibodies.

Samples of cerebrospinal fluid from 103 patients with meningitis were tested by PCR for detection of leptospires, and the results were compared with those of MAT and an ELISA for detection of immunoglobulin M (ELISA-IgM). Of these samples, 39.8 per cent were positive by PCR and 8.74 per cent and 3.88 per cent were positive by MAT and ELISA-IgM respectively (Romero *et al.*, 1998).

Experimentally infected bovine semen samples were analyzed by PCR followed by RFLP analysis for the detection of *Leptospira* sp. Eighty per cent of semen samples were positive by PCR and the detection limit of PCR was 100 bacteria per ml of semen (Heinemann, 1999).

Wagenaar *et al.* (2000) found that sensitivity of Nucleic acid hybridization was 55 per cent, culture 89 per cent and immunofluorescense (IF) was 93 per cent for detection of *L . borgpetersenii* serovar *hardjo* in bovine urine. A single technique was not sensitive for each animal tested. Therefore, the use of two techniques in combination was warranted for maximal sensitivity for diagnosis.

Venkatesha *et al.* (2001) compared the molecular techniques such as nucleic acid hybridization using slot blot and PCR with routine diagnostic method, and found that the slot blot hybridization detected 76.83 per cent cases within two day and PCR detected 81.7 per cent of the samples tested within one day.

Senthilkumar *et al.* (2001a) evaluated usefulness of PCR for rapid diagnosis of leptospirosis. In case of cattle, percentage of positivity by DFM was 50 per cent, 33.33 per cent and 50 per cent in blood, urine, milk respectively. But in PCR it was 75 per cent, 33.33 per cent and 50 per cent, respectively.

Senthil Kumar *et al.* (2001b) described a simple and rapid method for isolation of good quality DNA from leptospiral cultures using high salt method, which was cost effective and avoided use of enzymatic chemicals like protease. Lysozyme had been included in this procedure to lyse the bacterial cell wall. The DNA thus prepared could be directly used for the molecular biological works such as restriction enzyme digestion, PCR and RAPD methods.

Ramdass *et al.* (2002) adopted AP-PCR for identification of laboratory strains of leptospires and leptospiral cultures at serovar level. It was a simple and rapid method for typing leptospiral serovars.The main advantage of this technique was that culture sample could be directly used for typing, thus enabling characterization of leptospires at serovar level without need for DNA preparation

Nassi *et al.* (2003) used nested PCR for the diagnosis of leptosprosis. Primers were designed to amplify a 264 bp region with a *Lip L32* gene. The sensitivity and specificity of the assay were evaluated using seven saprophytic serovars and 35 pathogenic serovars. In order to enhance the sensitivity, another primer pair was designed which amplified a 183 bp region within 264 bp region in *Lip L32* gene and used in a nested PCR assay.

According to Martuce *et al.* (2003) low stringency single specific primer PCR (LSSP-PCR) was a rapid, simple technique that detected sequence variation in DNA fragments by amplification under very low stringency condition, with a single primer specific for one of the extremities of the template. It was performed to detect DNA polymorphism in a 285 bp DNA fragment amplified from genomic DNA with G_1 and G_2 selected primers.

Roy *et al.* (2003) compared the leptospires isolated from the Andaman in 1929 with one of the earliest available isolates from these islands, to study their serological and genetic relatedness. Randomly Amplified Polymorphic DNA (RAPD) finger prints of the isolates revealed that some of the recent isolates were genetically identical to the 1929 isolate.

Elaiyaraja. (2003) compared the efficiency of PCR with DFM and culture in the diagnosis of leptospirosis and found PCR to be more sensitive, specific and rapid over conventional methods, as it detected 41.6 per cent compared to 25.6 per cent by DFM and 2.4 per cent by culture of sample tested.

Alberto *et al.* (2004) found that *Leptospira biflexa* makes the isolation and characterization of pathogenic leptospires difficult and troublesome. To overcome this difficulty they used double set of primers (G_1/G_2, B64-1/B64-11) to amplify the DNA fragment of *Leptospira interrogans.* This primer was also sensitive to detecte even 10 micro organisms and could not amplify DNA samples from 29 saprophytic strains belonging to *Leptospira biflexa.*

Dey *et al.* (2004) described *Lip L32*, as a major leptospiral outer membrane protein (OMP), expressed only in pathogenic leptospiral species and was conserved among 200 serovars of *Leptospira.* The forward and reverse primers corresponding to *Lip L32* gene specific for the pathogenic serovars of *Leptospira* were used as diagnostic markers for screening human and canine leptospiral infection.

Sugathan and Vargheese. (2005) described Multiplex PCR using two sets of primers G_1 ,G_2 and B64-I , B64- II, giving specific amplification in 285bp and 563 bp regions respectively, by which strains could be detected rapidly. In this case, G1 and G2 were derived from the genomic library of *Interrogans* strain RGA, and this clone will react with all pathogenic leptospires except *L.Kirschneri* . The other set B64-I and B64-II were derived from the genomic library of *L. kirschneri.* All the isolates giving an amplicon of 285 bp belonged to serogroup Grippotyphosa and all the isolates giving an amplicon of 563 bp were assigned to *L.kirschneri* genomospecies.

Dhannia (2005) attempted to detect and differentiate leptospires in biomaterials using different PCR techniques. One hundred and forty seven clinical samples were subjected to genus specific PCR out of which nine were positive for *Leptospira.* These were also amplified by Nested PCR, whereas Multiplex PCR, LSSP-PCR, and AP-PCR failed to produce detectable amplification, probably due to low number of organism in bio materials. Among the different PCR technique used in the study LSSP-PCR was found to be more useful to differentiate leptospiral serovars.

2.7.4.2 Restriction Enzyme Analysis (REA)

Marshall *et al.* (1981) examined strains of *L interrogans* by REA. There was no detectable variation in the *EcoR1*endonuclease finger prints between isolates from different species of animals and of the same species. Although *EcoR1*endonuclease did not reveal difference between different strains belonging to the same serovar other endonuclease might detect minor variation.

Robinson *et al.* (1982) compared various strains of *Leptospira interrogans* by BRENDA. Field strains of serovar *hardjo* differed strikingly from the *hardjo* reference strain. Similarly subdivision of these serovars into distinct subtypes as defined by BRENDA was therefore useful and justified.

Hathaway *et al.* (1985) attempted to differentiate reference strains of leptospires of the Pomona serogroup by cross agglutination absortion and by BRENDA. Bacterial restriction endonuclease DNA analysis (BRENDA) proved that *pomona*, *mozdok* 5621, *proechimys* LT 796 and *tropica* CZ299 produced dissimilar electrophoretic DNA patterns and were placed in separate groups. These are in agreement with serovar classification produced by cross agglutination absorption tests. However, *dania* K1 and *tsaratsova* B 81/7 produced electrophoretic pattern identical to *mozdok* 5621 Strains.

A new method of classification for leptospiral isolate belonging to serogroup Pomona were analyzed and compared with those of type strain by cleavage with REA. All the twenty five field isolates tested, regardless of animal species they were isolated from, their country of origin and degree of laboratory adaptation, showed identical REA patterns. This is suggestive of the stability of genome of organism (Theirmann *et al.* 1985).

Ellis *et al.* (1991) performed restriction endonuclease analysis on DNA from the type strains of the Australis sero group of *Leptospira interrogans* by using twenty restrictions enzymes and the electrphoretic patterns obtained were compared with patterns obtained from one hundred and sixty two Australis sero group isolated from pigs. It proved to be a quick and reliable method of typing such strains.

Genetic variability among *L.borgpetersenii* serovar *hardjo type* Hardjobovis isolated from several geographical regions were determined by REA and by dot blot hybridization. Pulsed field gel electrophoresis of large restriction fragments generated by rare cutting enzymes suggested that some of the genetic differences among Hardjobovis isolates might result from genetic rearrangement (Zuerner *et al.*, 1993).

Savio *et al.* (1994) found the combination of PCR and REA of amplified products (PCR Restriction Fragment Length Polymorphism-RFLP) could be used to classify different serovars of *Leptospira interrogans* by detecting endoflagellin gene.

Senthilkumar *et al.* (1997) used REA for characterizing the various leptospiral serovars. Reference strains and rat isolate R1 were selected for this study. Samples of DNA were prepared and subjected to REA analysis with different enzymes, among the patterns produced by the enzyme *Hae 111* facilitated clear differentiation between different serovars. The rat isolate R1 restriction enzyme map was similar in pattern with the reference strain Veldrkt Batavia 46 of the serovar *javanica.*

Dhannia (2005) carried out REA of product of genus specific PCR using the enzyme *Dde 1.* Out of the 16 *Leptospira* cultures the serovars *rachmati, grippotyphosa* and *tarassovi* presented digestion patterns different from other serovars.Among these, digestion pattern of *grippotyphosa* and *tarassovi* were the same. The REA of 285 bp product of primers G1/G2 with the same enzyme yielded three different patterns out of the 14 Leptospira serovars. The serovars *grippotyphosa* and *tarassovi* presented unique digestion patterns whereas the other 12 serovars had same pattern.

2.9 BOVINE MYCOPLASMOSIS

Mycoplasma of cattle enjoy a special place in the history of *Mycoplasma* research, as the very first *Mycoplasma* known was the agent of contagious bovine pleuropneumonia (Gourlay and Howard, 1979). First *Mycoplasma* to be recognized as a cause of mastitis was *Mycoplasma bovigenitalium* reported from England in 1960 by Stuart. By far the most prevalent cause of *Mycoplasma* mastitis is *Mycoplasma bovis.* It was reported as a cause of mastitis in Connecticut in 1962 by Hale *et al.* at which time it was tentatively called *M.agalactiae var bovis* known also as *M.bovi mastitidis* (Jain *et al.*, 1967). It was

officially designated as *M.bovis* in 1976 (Aska, 1976). Gonzalez and Wilson (2003) listed other *Mycoplasma* sp. that have been associated with mastitis are *M.alkalescens, M. canadense. M.californicum* designated as ST-6 (Jasper 1977) *M.bovirhinis, M.argini, Mycoplasma* species group 7, *M.dispar, Ureaplasma urealyticum* and *Acholeplasma laidlawii.*Tully *et al.* (1993) reported that about 125 species of mycoplasmas have been recognized and they are currently classified into eight genera. Freundt and Edward (1969) proposed that the organism be assigned to the order *Mycoplasmatales*, class *Mollicutes*, family *Mycoplasmataceae* , genus *Mycoplasma.* Later genus *Ureaplasma* was recognized and two new families with one genus each, the *Acholeplasmataceae* and the *Spiroplasmataceae.*

2.10 PREVALENCE

Ruhnke *et al.* (1975) identified bovine mastitis in Ontario due to *Mycoplasma agalactiae* subsp. *bovis.*

Jasper (1977) reported five important serotypes of mycoplasmas isolated from cows with mastitis in California .They were *M.bovis M. bovigenitalium, M.alkalescens, , M. canadensis* and *strain ST -6* .Most reported occurrences were due to *M.bovis.*

Pal *et al.* (1982) isolated *Mycoplasma bovigenitalium* and *Acholeplasma laidlawii* from mastitic milk of a cow and buffalo in India. No other bacteria could be isolated from the samples from which *Mycoplasma* isolations were made.

DaMassa (1983) isolated *Mycoplasma agalactiae* and *M mycoides* subsp *mycoides* from the mastitic milk of a goat in California.

Kunkel (1984) collected 2346 bulk samples from Vermont dairy farms for the isolation of *Mycoplasma*.Three samples gave a positive isolation for *Mycoplasma bovis.*

From bulk tank milk cultures, Gonzalez (1988) isolated potential mastitic pathogens from 22 per cent of cows in California. *Staphylococcus aureus* was isolated from 50 herds, *Streptococcus agalactiae* from 47 herds, and *Mycoplasma* species from 24 herds.

Kumar and Garg (1990) isolated mycoplasmas from mastitic and healthy bovines in India.They found that the isolation rate of *Mollicutes* was higher in mastitic milk of cows and buffaloe than in the milk of normal cows and buffaloes.

Ghadersohi *et al.* (1999) detected *M bovis* in over 50 per cent of bulk milk samples with somatic cell count (SCC) $250x10^3$ cells/ ml, from dairy herds in North Queensland and Victoria.

Sickles (2000) isolated *M.bovis* and *Acholeplasma laidlawii* from composite milk samples from cows with clinical mastitis in Chile. *M. bovis* mastitis was diagnosed from bulk tank milk in Republic of Ireland (Byrene *et al.*, 2001).

Hirose *et al.* (2001) isolated *Mycoplasma alkalescens, M. bovigenitalium, M.bovirhinis* and *M.bovis* from milk specimen in Japan .

Nicholas and Ayling (2003) described *Mycoplasma bovis* as a major and often overlooked, cause of calf pneumonia, mastitis, arthritis and other conditions. This organism is found world wide and has spread to new areas including Ireland and parts of South America in the last decade.

Herd prevalence estimates by culture of *Mycoplasma* species in bulk tank milk suggested that between one and eight per cent of all herds had at least one cow with *Mycoplasma* mastitis. These prevalence estimates were all from the USA (Fox *et al.*, 2003).

2.11 PROPERTIES OF ORGANISM

Neimark (1967) found that *Mycoplasma* were prokaryotes, possessed a triple layer unit membrane, 70S ribosome and a typical prokaryotic circular chromosome .They are the smallest organisms known to be capable of growth in cell free media. Guanosine cytosine contents of *Mycoplasma* DNA is 23-40 mol per cent. Frenundt and Edward (1969) observed that *Mycoplasma* lacked a wall and were bounded only by a single membrane. Therefore the organism was classified as a member of *Mollicutes*.

The phylogenetic position of *M. bovis* with respect to other *Mycoplasma* was determined by sequence comparisons and from features in the secondary structure of 16S rRNA. Comparisons with 16S rRNA sequences of *Mycoplasmas* showed that only eight nucleotides differed between the *M. bovis* and *M. agalactiae* (Mattsson *et al.*, 1994).

Raizin *et al.* (1998) observed that simple genome and fastidious growth requirements of *Mycoplasma* could be associated with their slow replication and difficult identification in many mastitis diagnostic laboratories. Thus, many *Mycoplasma* mastitis infections might be under diagnosed.

Hayman and Hirst (2003) provided evidence that *M.bovis* was host specific and that most cattle were colonized as a part of their normal flora.

Nicholas and Ayling (2003) found that *M.bovis* was highly adapted to cattle but occasional isolations had been made in buffaloe, small ruminants and even humans.

2.12 EPIDEMIOLOGY OF MYCOPLASMA INFECTION

2.12.1 Reservoir and Source of Infection

Jasper (1977) found that natural resolution of the disease occurs, but timing of natural resolution was highly unpredictable. With natural or therapeutic clinical recoveries cow might intermittently shed *Mycoplasma* in nearly normal milk for a few weeks or months. These cows represent a danger to other cows in the herd.

Bushnell *et al.* (1984) postulated that *Mycoplasma* species was frequently present in the population of dairy cattle and therefore, would invade the mammary gland and cause mastitis.

Jasper (1988) opined that cows that recovered clinically tend to be intermittent shedders of small numbers of organisms for an indefinite period. Apparently recovered cows might be a danger to other cows in a herd and hence their retention in the main herd was undesirable.

Sickle (2000) observed that dairy cattle of different ages might be asymptomatic carriers of *Mycoplasma* .Several species of these micro organisms were part of the natural flora of respiratory and urogenital tracts of these animals.

Some cows were intermittent shedders of *Mycoplasma* sp. in the milk, with as many as fourteen per centage of the cows shedding less than 100 CFU/ ml of this organism from infected glands (Biddle *et al.*, 2003).

Fox *et al.* (2003) suggested that as larger herds tend to purchase more replacements, the risk of eventually acquiring an animal with intra mammary *Mycoplasma* infection would be greater in larger herds. Large herds that carried *Mycoplasma* either as intra mammary infections or as colonization or infections at other organ site, contributed to *Mycoplasma* positive herds status.

Gonzalez and Wilson (2003) suggested that most often transmission occurred from replacement animals that were introduced into the farm.

2.12.2 Mode of Transmission

Jasper *et al.* (1966) noted that in one herd with *Mycoplasma* outbreak, cows were also suffering from *Mycoplasma* arthritis, suggesting that disease agent was systemically transferred. Additionally, some cows had no signs of clinical mastitis but shed *Mycoplasma* species and leucocytes in milk in abnormally high levels.

Hamdy and Trapp (1967) found that bovine *Mycoplasma* might have been transported within the cows either by lymphatic system or blood circulation.

Spread of *Mycoplasma* mastitis from cows to cow in infected herds was via streak canal while quarter to quarter spread was often systemic rather than through streak canal. Infected aerosol droplets could play a role in spreading *M.bovis* from cow to cow (Jasper, 1977).

Bennet and Jasper (1978) observed that *Mycoplasma* infection was spread from one quarter to another via haematogenous route.

Bushnell (1984) stated that *Mycoplasma* mastitis might come from mechanical transfer of respiratory and urogenital tract infections to the mammary gland.

Gonzalez *et al.* (1993) suggested that transmission of *Mycoplasma* infections between animals occurred via nasal discharge. Thereafter, transfer of *Mycoplasma* species from the respiratory carriage site to the mammary gland or skeletal joints were observed.

Pfutzner and Sachse (1996) reported that there was horizontal and vertical transmission for *M.bovis* associated with mastitis and carriage to other body sties.

Fox *et al.* (2005) reported that a significant number of *Mycoplasma* outbreaks might occur endogenously or animal to animal transmission of *Mycoplasma* mastitis pathogens from asymptomatic carriers.

2.12.3 Association with Other Organism

From cow composite milk samples Gonzalez (1988) found that the prevalence were 9.3 per cent for *Streptococcus agalactiae*, 9.1 per cent for *Staphylococcus aureus*, 0.9 per cent for *Mycoplasma* species, 1.2 per cent for coliform bacteria, 0.9 per cent for other streptococci , 0.8 per cent for coagulase negative staphylococci and 1.3 per cent for other organisms.

Ghadersohi *et al.* (1999) proposed that bacteria such as *Streptococcus aglactiae*, *Staphylococcus aureus* and other bacterial species could be considered as secondary pathogens acting synergistically with *M. bovis* in the development of mastitis.

Mycoplasma bovirhinis was often isolated from respiratory organ. *M.bovirhinis* was detected with high frequency in clinical mastitic milk specimen was probably due to infection caused by secretions of calves through nipples simultaneously contaminated by other organism during lactation (Hirose *et al.*, 2001).

Fox *et al.* (2003) opined that the presence of other contagious mastitic pathogens, *Staphylococcus aureus* and *Streptococcus agalactiae*, was not related to the presence of *Mycoplasma*, suggesting that the etiology and transmission of *Mycoplasma* mastitis were different from transmission of other contagious mastitis pathogens.

2.12.4 Pathogenesis of Mycoplasmosis

Mycoplasma bovis antigens appeared to be immuno suppressive since lymphocytes from calves inoculated with *M.bovis* antigen had reduced lymphoblastogenesis (Bennnet and Jasper, 1977).

The *Vsps* genes have multiple functions in *M.bovis*. They helped in the adhesion to the host cell, to evade the immune system by frequent variations and were adapted to different conditions in the host and environment (Sachse *et al.*, 1996).

Thomas *et al.* (2003) found that *Mycoplasma* isolated from clinical mastitis cases exhibited high adherence rate when compared to non pathogenic strains. Cytadhesion was assumed to be a crucial step in the initial phase of infection, as attachment of *M.bovis* to specific receptors on epithelial cells in the respiratory tract was an essential prerequisite for colonization of host tissue.

The mechanisms of the pathogenesis of *Mycoplasma* were still relatively unknown although it was clear that it used complex strategies to invade the host organism. Adhesion of the *Mycoplasma* to the host cell was the primary and key factor of pathogenesis (Tenk 2005).

2.12.5 Clinical Signs

2.12.5.1 Mastitis

The clinical signs of *Mycoplasma* mastitis were similar in all the affected animals. Approximately 24 hours after the initial drops in milk production, there was a slight thickening of the milk with few flakes. Then the secretion gradually became purulent with large clots which settled out leaving a watery supernatant. This type of milk was characteristic of the condition. No blood was observed and there was no odour.The swelling of udder was not very

noticeable. Temperature and appetite remained normal during the infection. None of the affected cows responded to treatment with any of an extensive range of intramammary preparations. The poor management practices were not always a factor since the disease also occurred in herds where management was good or above average (Ruhnke *et al.,* 1975).

Bennet and Jasper (1977) observed that *M.bovis* mastitis was characterized generally by acute onset and progression to severe chronic mastitis.

Jasper (1977) observed that *Mycoplasma* mastitis was characterized by purulent mastitis that resisted treatment. However, cows continued to eat and had very little evidence of illness, except an early transitory fever which usually escaped detection. Mastitis which typically involved more than one quarter (often all quarters). Marked loss of production was there in affected cows. Most of the affected cows showed severe mastitis, but some might simply cease lactating. Abnormal secretions with tannish or brownish discolouration, sandy or flaky sediments in watery or serous fluids were also noticed. Particles might rise to top of a tube or sinked to bottom. After a few days, the entire secretion might be purulent or serous.

Experimental inoculation of 70 CFU/ml of organisms into a non infected quarter could produce a severe purulent infection. The presence of large numbers of *Mycoplasma* exceeding 10^6 CFU /ml of milk preceded the onset of overt sero purulent mastitis by one to three days (Bennet *et al.*, 1978).

Ghadersohi *et al.* (1999) reported the association of *M. bovis* with elevated somatic cell count(SCC). Milk showing chronically high milk SCC in *Mycoplasma* infected herds suggested that this agent might play an important role in the etiology of sub clinical and chronic mastitis.

Some large dairy farms in southern Chile had been suffering with high incidence of clinical mastitis refractory to antibiotics treatment, with low bulk

milk somatic cell count and negative results on conventional culture media (Sickle *et al.*, 2000).

2.12.5.2 Genital Infection

Edward *et al.* (1947) isolated *Mycoplasma bovigenitalium* and *Acholeplasma laidlawii* from infertility cases of cattle in England.

Balita (1987) demonstrated a high incidence of *M .bovis*, *M. canadense, M californicum*, *M. alkalescens* and *Ureaplasma* contamination of bovine semen collected for use in artificial insemination. He isolated *M californicum* from fresh semen sample and also from the vagina of cows with a *M. californicum* mastitis, indicated that semen could be a means of distribution of the organism and the male and female genital tracts were potential reservoirs of the infection.

Ulgen *et al.* (2003) isolated *Mycoplasma bovigenitalium .M.bovis* and *M.mycoides* subsp. *mycoides (Small Colony)* from the bulls among which *M. bovigenitalium* was prevalent from the preputial washing of bulls slaughtered at Bursa province, Turkey.

2.12.5.3 Respiratory Infection

Langford (1977) isolated *M.agalactiae* subsp. *bovis* in pure culture from pneumonic lungs of cattle indicating that this species by itself might have a role in the pathogenesis of pneumonia. However, some or all of these animals had a prior infection with either some other bacteria or viruses capable of inducing an inflammatory response. The *Mycoplasma* invaded the damage tissue after the initial invader had been controlled by the host defense system and or treatment. The failure to recover *Mycoplasma* species from the normal lung was further evidenced that this organism should be considered as a possible etiological agent of pneumonia in cattle.

Gourlay and Howard (1979) reported that *M.bovirhinis* was a trouble some agent acting as a secondary invader in respiratory diseases, because the *Mycoplasma bovirhinis* was most commonly isolated from the nasal cavity of cattle with respiratory diseases.

Mycoplasma bovis was isolated from fatal pneumonia of cattle in Republic of Ireland (Byrne *et al.*, 2001).

2.12.6 Duration of Infection

Hamdy and Trapp (1967) found that bovine *Mycoplasma* might require a longer incubation period under favorable conditions to induce lesion of a chronic nature.

Bennet and Jasper (1977) reported considerable variation in the duration *M. bovis* mastitis which ranged from 15 days to more than 100 days.

Jasper (1977) found that recovery from mycoplasmal mastitis was slow. Shedding of *Mycoplasma* into milk of apparently recovered cows for an indefinite period was common.

Fox *et al.* (2003) opined that in herds that remained positive for *Mycoplasma,* the concentration in the bulk tank remained relatively constant over at least the first six months after first isolation. The agent was not found in some herds tested repeatedly after the initial positive isolation, which was strong evidence of clearance.

2.12.7 Immunity in Mycoplasmosis

Howard (1976) reported that IgG active against *M.bovis* was necessary for successful phagocytosis by macrophages and neutrophils. Consequently serum anti *M.bovis* IgG played an important role in the immune response to *M.bovis* mammary infections.

Locally produced antibody prevented attachment of *Mycoplasma* to the bronchial epithelium, inhibiting growth of organism, killing mycoplasmas directly with or without complement or by promoting their phagocytosis. There was no direct relationship between serum antibody level and immunity to respiratory infection (Howard *et al.*, 1977).

Bennet and Jasper (1977) reported that the cell mediated immune response might be necessary for resolution of mycoplasmal mastitis both directly and via their helper cell function on antibody production. Successful containment and resolution of udder due to *M.bovis* appeared to be largely dependent on the ability of infected host to mount a local and systemic immune response.

Jasper (1977) found that it was not unusual to find infected cows which had no history of mastitis. This might be related to partial immunity from a prior non masitic exposure to *Mycoplasma* or infections in the respiratory, reproductive or other system.

The first response to infection with *M. bovis* was a depression of the immune system, as measured by lymphocyte stimulation test. Strong cell mediated responses were not detected until forty fourth day following experimental inoculation of organisms into calves (Bennet and Jasper, 1977).

Bennet and Jasper (1978) found that recent prior infection did not assure resistance to reinfection.

In lactating cows milk IgA and IgG concentration were higher in previously infected quarters than in other quarters and also were associated with resistance to infection (Bennet and Jasper 1978) .

Bennet and Jasper (1980) observed that Milk whey IgG and IgA concentration increased in quarters that resolved the infection. The mechanisms that control and enhance IgA production were not well understood, might be

related to successful T-cell lymphocyte activities. Hence the total quantity of IgA and possibly IgG, secreted daily by lactating mammary tissues might be more critical to the resolution than their actual concentrations.

Jasper (1981) reported eosinophils rather than neutrophils to be the basic inflammatory cells in tissues and alveoli infected with *M.bovis*. He also found that a pre existing leukocytosis did not protect quarters against mycoplasmal infections. An immunological resistance was suspected in infected herds which were shedding the organism without a history of clinical mastitis.

Jasper (1988) suggested that in herds having the problem of mastitis over a period of years, a degree of resistance seemed to develop on a herd wide basis. So that severe cases of mastitis were less common.

2.13 DIAGNOSIS

2.13.1 Culture and Isolation of Mycoplasma

Jasper (1977) opined that use of composite samples was satisfactory to diagnose the mycoplasmosis. He also found that a significant number of apparently normal cows were shedding small numbers of *Mycoplasma* into the milk.

Acholeplasma laidlawii isolated from mastitis milk was considered as a common contaminant of cow's environment (Gourlay and Howard, 1979).

T.Shimizu (1983 found that the tween serum plate serve as a selective medium for the rapid and reliable isolation of *M bovis* because it will completely inhibit the growth of *M. bovirhinis*, which is the most frequent contaminant *Mycoplasma* species in nasal discharges of calves.

Presence of $6.3x10^7$ CFU/ml of mycoplasmas in the mammary secretion indicated active infections (DaMaasa, 1983).

Kunkel (1984) opined that bulk tank culturing might be helpful in monitoring for the presence of the *Mycoplasma* infection.

The risk of large herds (> 700 cows) having a *Mycoplasma* positive bulk tank sample was eleven times greater than that of a small herd < 350 cows. (Jasper, 1988).

Kumar *et al.* (1990) opined that the isolation rate of *Mollicutes* was higher in mastitis milk of cows (15.38 per cent) and buffaloe (5.05 per cent) than in the milk of normal cows (7.58 per cent) and buffaloe (0.98 per cent). Mastitic cows (3.8 per cent), normal cows (2.7 per cent) and mastitis buffaloe (2.08 per cent) were *ureaplasma* positive. But naturally occurring mastitis in cows due to *Ureaplasma* was rare. *A. laidlawii* was isolated from healthy and mastitic cows.

Kirk *et al.* (1997) reported that prevalence of positive samples varied from 1.8-5.8 per cent for all species of *Mycoplasma* and from 1.22-3.1 per cent for *Mycoplasma* species known to be mastitis pathogen. *Mycoplasma bovis, M.californicum,* and *M bovigenitalium* were consistently isolated, but *M. bovis* was the most commonly isolated species.

Ghadersohi *et al.* (1999) observed some animals were intermittently shedding *M. bovis* because the organism was detected at intervals of one month in most samples. So frequent testing would be required to detect all animals infected with *M. bovis.*

Kaur and Garg(2000) reported that the isolation rate of *Mollicutes* was higher from mastitic milk of cows (47.5 per cent) and goats (11.4 per cent) than from the milk of normal cows (21.7 per cent) and goats (2 per cent). Isolation rates of *Ureaplasma* were 29.1 per cent, 23.8 per cent and 11.4 per cent from

mastitis cows, buffaloe and goats respectively. However ureaplasmas were also isolated from normal milk of 14.4 per cent, 30.9 per cent and 2 per cent cows, buffaloe and goats respectively. So far only one species, *U. diversum* was known to occur in bovines.

The isolation of *M.bovis* from milk sample was the definitive confirmation of *M.bovis* mastitis. The sensitivity of these culture based methods of detection depended on the continuous, excretion of *M.bovis* from any infected quarter, the aseptic collection of milk from each quarter and the survival of *Mycoplasma* under the conditions of transit in sufficient numbers to be detected (Byrene *et al.* 2000).

Culture of bulk tank milk sample had been advocated as a primary screening method to determine *Mycoplasma* status of a herd. But sensitivity of detection of *Mycoplasma* species in bulk tank milk was affected by a significant minority of cows that might shed the organism at levels below the threshold of detection. Completion of culture for *Mycoplasma* organism could take ten days (Fox *et al.*, 2005)

Richard *et al.* (2006) reported that bulk tank milk culture might be used as a monitoring tool in the control and evaluation of clinical and sub clinical mastitis because bulk tank milk culture was a cheap and convenient method of evaluating milk quality compared with collection and culturing of individual cow milk sample. And, it might be a useful tool for estimating herd level prevalence of contagious mastitis pathogen.

2.13.2 Serology

Jain *et al.* (1969) determined that cattle would mount an immune response to bovine mycoplasmosis by producing detectable levels of antibody, and thus serology had been used in diagnosis. While interpreting the serology,

care must be taken that detection of antibodies in an animal implies exposure not disease. Risk of a sero positive test increased with age and with farm size.

Howard *et al.* (1976) described a radial growth precipitation test for measuring antibody to *Mycoplasma aglactiae* subsp. *bovis*. The test was quantitative and appeared to depend the production of soluble antigen by growing organism. It was found to have sensitivity comparable to that of complement fixation test.

Jasper (1977) found that Indirect Haemagglutination Test(IHA) tests appeared to be more sensitive and technically more reliable and mean titers were high in infected or previously infected animals compared with low titers in animals not exposed to *M.bovis*. Titer was not a sure guide to current infection because some animals with high titers might have recovered, and recently infected animals might not yet show a titer.

Bennet and Jasper (1977) opined that elevated IHA titers, serum growth inhibitory activitity,and immediate skin reactions to intra dermally injected viable or non viable *M.bovis* organisms were typical of cows with either natural or experimental *M.bovis* mastitis. They found that presence of locally secreted antibody and locally active immune cells could provide a better indication of those animals in the process of resolving the infection than was observed using systemic indicators of immune responsiveness such as indirect haemagglutination or growth inhibition tests.

Experimental inoculation of 70CFU /ml of *Mycoplasma bovis* strain L47 proved that IgM was predominant immunoglobulin responsible for serum indirect hemagglutination titers. After 57 days, IgG was predominant. Milk whey indirect hemagglutination titers did not distinguish between quarters resolving the infection and those that did not (Bennet, 1979).

Kotani et *al.* (1986) developed an Immuno Binding Assay (IBA) to identify *Mycoplasma* colonies on agar in pure and mixed cultures. . The IBA was used to screen for monoclonal antibodies and for rapid detection of *Mycoplasma.* Immuno Binding Assay was reported as an alternative to ELISA.

Boothby *et al.* (1986) used monoclonal antibodies for the detection of *M.bovis* antigen in milk. They observed that the sensitivity of monoclonal antibody test will approach 1000 CFU per ml of *M.bovis* and it could identify species specific antigen.

Ball *et al.* (1990) reported a sensitive test using a monoclonal antigen capture ELISA test using 1000CFU/ml *M. californicum* . They reported the test was highly specific. However, the speed and ease and the accuracy of the ELISA and imunobinding tests using monoclonal antibodies as described made them worthy of consideration as a monitoring or screening tool.

Ayling (1997) reported that *M.bovis* possessed a series of prominent membrane lipoproteins on the cell surface. This variable surface lipoprotein might also have an effect on the reduced ability of diagnostic laboratory to identify some *M.bovis* isolates by conventional serological methods, such as the film inhibition test and immunofluorescent antibody tests.

Sickles (2000) differentiated *Mycoplasma* to species level by an indirect immuno peroxidase test.

Indirect ELISA might be useful for the detection of cows which had recently developed *M.bovis* mastitis. *Mycoplasma bovis* specific immunoglobulin appeared in the milk within first nine weeks of outbreak. So Indirect ELISA could be applied as a screening test to the milk sample collected from any herd affected by *M.bovis* mastitis (Byrne *et al.,* 2000).

Using a monoclonal antibody (MAb) raised to a *M .bovis* antigen, a sandwich ELISA was developed for routine detection of the organism. The sensitivity of sandwich ELISA was improved by combining enrichment with the initial capture stage, by titrating test samples (10^1-10^3) in *Mycoplasma* medium in microtitre wells coated with MAb (Brice *et al.* 2000) .

Byrne *et al.* (2000) reported the use of an Indirect ELISA to detect *M. bovis* antibodies in milk.The test had 92 per cent specificity and 100 per cent sensitivity. Such milk antibody could be used as a screening test for *Mycoplasma* mastitis and further confirmed by culture results.

Fox *et al.*(2005) suggested that examination of *Mycoplasma* antigens in milk as a diagnostic test for *Mycoplasma* mastitis would seem to have greater value than diagnosis by antibody detection as the later test was inherently weakened by the reduced ability to distinguish between current and previous infection or exposure to the agent.

Ghadersohi *et al.* (2005) developed a monoclonal antibody blocking enzyme linked immunosorbent assay (B- ELISA) to detect antibodies to *Mycoplasma bovis* in cattle sera. The assay was highly specific and sensitive and there was no cross reaction detected. There was a strong positive correlation between PCR and B- ELISA titers. Thus the B ELISA appeared to be a valuable and reproducible tool in the sero diagnosis of *M. bovis* infection in cattle.

Robino *et al.* (2005) demonstrated membrane lipoprotein homologous to the P48 of *Mycoplasma agalactiae* in different *M. bovis* isolates. This protein was structurally and antigenically conserved within the *M. bovis*. The *p48* gene was identified by PCR approach and partially sequenced. The purified recombinant antigen (r-P48) was evaluated as a potential marker of *M.bovis* infection.

2.13.3 Molecular Methods

Jasper (1981) opined that *Mycoplasma* infection might or might not produce an inflammatory reaction. Thus SCC could not detect all udders infected with *Mycoplasma*. Hence, any control programme of *M. bovis* infection should use PCR to detect infected animals.

Hotzel *et al.* (1993) developed a PCR for diagnosis of *M.bovis* in milk samples. Their procedure included trypsin treatment of milk with detergent followed by passage through a DNA binding filter. The DNA was then extracted and nucleic acid fragments were amplified by PCR. Before southern hybridization the sensitivity was 500 CFU/ml and increased 10-fold after hybridization. Moreover, results were obtained within 24 h.

Polymerase chain reaction was a powerful and valuable tool for ensuring the correct identification of *Mycoplasma* isolates. In this method only small numbers of organism were required to enable rapid identification of isolates and potential problems associated with some serological identification tests might be avoided (Ayling, 1994).

Gonzalez *et al.* (1995) designed a molecular primer to amplify 16S rRNA genes of *M.bovis* and *M.agalactiae* and developed two different PCR system based on 16S rRNA sequences to detect *Mycoplasma* in animals with sub clinical or chronic disease. The detection limit of PCR system for *M bovis* with reference culture was $4x10^2$ CFU/ ml and of the PCR system for *M agalactiae* it was $2x10^2$ CFU /ml.

Ayling *et al.* (1997) confirmed that compared to conventional methods such as indirect ELISA or serological identification of *Mycoplasma* isolates using film inhibition test, the sensitivity of PCR assay was very high. Only small numbers of organism were required to enable rapid identification of isolates and

potential problems associated with some serological identification were overcome by PCR.

Ghadersohi *et al.* (1997) compared the sensitivity of dot blot hybridization assay with *M.bovis* specific PCR, for the identification of *M. bovis.* The level of sensitivity of dot blot hybridization was 200 CFU/ ml .The minimum amount of target DNA that could be detected by the PCR assay was 10-20 CFU/ml.The PCR assay was therefore ten times more sensitive than dot blot hybridization.

Kobayashi (1998) developed a PCR assay based on *in vitro* amplification of the 16S rRNA gene for the detection of *M. alkalescens, M. bovigenitalium and M. bovirhinis.* The sensitivity of each PCR system to detect *Mycoplasma* was 10^4 CFU/ml when a pure culture was tested and 2 x10^4 CFU/ ml in diluted nasal mucus solution. *Mycoplasma bovirhinis* was the organism frequently isolated from the nasal swabs using an enrichment culture medium.

Mycoplasma bovis PCR assay (MB-PCR) had been reported to have high sensitivity and specificity. . The sensitivity of the MB-PCR was approximately 30 CFU/ml of milk.This could detect animals with a very low level of the infection, indicating that the organism might be carried asymptomatically by animals with a low SCC (Ghadersohi *et al.*, 1999).

Butler *et al.* (2001) used AP- PCR to investigate *M.bovis* outbreaks in cattle .The AP- PCR typing method provided genotypic epidemiological information to successfully characterize *M. bovis* from sequential sampling of outbreaks and different husbandry conditions.

Pinnow *et al.* (2001) reported excellent success in detection of *M.bovis* in milk sample preserved by a thioloxidant using a Nested PCR. The Sensitivity of PCR was 5.1 CFU /ml and 100 per cent specific.

Hirose (2001) developed a PCR assay based on *in vitro* amplification of 16S rRNA gene for the detection of *M.alkalescens, M. bovigenitalium, M.bovirhinis* and *M. bovis.* He found that detection limit of the *Mycoplasma* culture method was almost equal to those in PCR or tended to be lower in PCR for all mycoplasmas.

Hayman *et al.* (2003) described Semi Nested PCR (SN-PCR) to detect *M. bovis* directly from clinical materials. The nonspecific banding produced in the single step PCR, was eliminated with the development of SN configuration, the end point for detection of *M. bovis* DNA in an extracted clinical sample was 91.5 picogram/microlitre after the first round of amplification. This was increased by 10 fold to 9.15 picogram per microlitre after the second round of amplification.

McAuliffe *et al.* (2003) developed the ability to distinguish between several different species of *Mycoplsma* from several different sources using denaturing gradient gel electrophoresis of a 16S rRNA PCR product. They were able to successfully differentiate between 27 of 32 species using their technique. Use of PCR had an advantage in that through the study of unique genomic constructs, the technique could be used in studying epidemiology of disease.

Das *et al.* (2003) carried out PCR using *M bovis* specific primers PpMB 920-1 and PpMB 920-2(Hotzel *et al.*, 1993) and yielded an amplified product of 2 kb, without any cross amplification with other *Mycoplasma* and *Acholeplasma.*

Alber *et al.* (2004) opined that the PCR technology had the advantage of ability to speciate mastitis pathogens.

Tenk (2005) designed PCR assay specific to detect *M. bovis,* yielding a 319 bp product. It neither amplified other bovine *Mycoplasma* including *M. agalactiae* nor the most frequent bacteria species. This assay was able to detect

150 CFU/ ml of organism in broth culture and could also detected *M bovis* in milk.

Deoxy ribonucleic acid based detection methods offered advantage in terms of specificity and sensitivity. Isolate from non typical hosts ie., *M. bovis* strains from small ruminants and *M. agalactiae* strains from cattle, were characterized by sequencing the 16S and part of the 23S ribosomal RNA genes (Bashiruddin *et al.*, 2005).

Cai *et al.* (2005) developed a Real Time PCR (RT-PCR) for detection of *Mycoplasma bovis* in bovine milk and lung samples. The detection limit was 550 CFU /ml of milk and 650 CFU/25 mg of lung tissue. The relative sensitivity and specificity were 100 per cent and 99. 3 per cent for individual milk and 96.6 per cent and 100 per cent for lung tissue. Real Time PCR assay was sensitive, specific and rapid method to identify *Mycoplasma bovis* infection in bovine milk and pneumonic lungs.

Materials and Methods

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1 *Leptospira*

3.1.1.1 Sample Collection

Milk samples were collected from suspected cases of bovine leptospirosis for PCR and for isolation and identification of organism .

3.1.1.2. Reference Strain of Leptospira

Reference strain of *Leptospira pomona* procured from National Leptospirosis Reference Centre, Regional Medical Research Centre, Port Blair, Andaman and Nicobar Islands, India and maintained in the laboratory was used for the study.

3.1.1.3 Phosphate Buffered Saline (PBS) stock solution (10 x)

Sodium Chloride	80 g
Potassium Chloride	2 g
Disodium hydrogen phosphate	11.32 g
Potassium dihydrogen phosphate	2 g
Distilled water	100 ml

The pH was adjusted to 7.4 by I N NaOH and sterilized by autoclaving at 121^{0}C for 15 min at 15 lbs pressure. The stock solution was diluted to 1x before use.

3.11.4. Reagents for PCR

3.1.1.4a Primers

Genus specific primers designed by Merien *et al.* (1992) were used for detection of leptospires in milk samples.

A: 5'-GGC GGC GCG TCT TAA ACA TG-3'

B: 5'-TTC CCC CCA TTG AGC AAG ATT-3' (IDT, USA).

3.1.1.4b PCR Reaction Buffer (10 x)

This includes 750mM Tris HCL P^H 8.5 ,200mM $(NH_4)\ SO_4$,, 15mM $Mgcl_2$, 1% Tween 20 (Genei, Bangalore).

3.1.1.4c Magnesium Chloride

Magnesium Chloride with strength of 25mM (Genei, Bangalore).

3.1.1.4d *Taq* DNA polymerase

The *Taq* DNA polymerase enzyme with a concentration of three units per microlitre (Genei, Bangalore).

3.1.1.4e Deoxy Nucleotide Triphosphates

Deoxy Nucleotide Triphosphates (dNTP mix) (2.5mM of each dGTP/dCTP/dATP/dTTP) (Genei, Bangalore).

3.1.1.5 Materials for submarine agarose gel electrophoresis

3.1.1.5a Agarose

Agarose low EEO (Genei)

3.1.1.5b Tris Acetate EDTA buffer

Stock solution (50X) (Genei)

1x solution prepared in distilled water was used for electrophoresis.

3.1.1.5c EDTA stock solution (0.5 M) pH 8.0

Sodium EDTA $2H_2O$	186.1g
Distilled water	800 ml

The pH was adjusted to 8.0 with 1 N NaOH .Distilled water was added to make up the volume to one litre. The solution was sterilized by autoclaving at 121^0C for 15 min at 15 lbs pressure.

3.1.1.5d Tris Borate EDTA Buffer (TBE) pH 8.2

a) Stock solution (10 x)

Tris base	108.0 g
Boric acid	5.0 g
EDTA (0.5 M, pH 8.0)	40 ml
Triple distilled water to make	1 litre

b) Tris Borate EDTA Buffer (TBE) 1x

TBE stock solution	10 ml
Triple distilled water to make	100 ml

3.1.1.5e Ethidium Bromide stock solution

Ethidium Bromide	10 mg
Triple distilled water	1 ml

The solution was mixed well and stored in amber colored bottles at 4 ^{0}C.

3.1.1.5f Gel Loading Buffer (6 x)

a) Sucrose solution

Sucrose	40 g
Triple distilled water	100 ml

b) Tracking dyes

Bromophenol Blue	0.25 g
Xylenexylol	0.25 g

The gel loading buffer was prepared by carefully dissolving the tracking dyes in sucrose solution and kept at 4° C.

3.1.1.5g DNA Molecular size marker

a) pUC 18/*Sau 3A 1*- pUC 18/*Taq 1* Digest with fragments of 1444, 943, 754, 585, 458, 341, 258, 153, 105 and 78 /75 base pairs.

b) pUC *19 DNA /Msp* I Digest consisting of 9 double-stranded DNA segments of 34/ 34, 67, 111/110, 147, 190, 242, 331, 404 and 501/489 base pairs.

3.1.2 METHODS FOR DETECTION OF *Leptospira*

3.1.2.1 Standardization of molecular techniques employing the reference strains

3.1.2.1.1 Maintenance of *Leptospira*

Stock cultures of reference strains of *Leptospira* isolates were maintained in Fletcher's semisolid media (3.2.5a) in BOD incubator at 28^0 C. The cultures were sub cultured routinely at four weeks interval. The purity of cultures was assessed by Dark Field Microscopy.

3.1.2.1.2 Polymerase Chain Reaction (PCR)

3.1.2.1 .2a Preparation of DNA template from stock culture

A drop of culture was added to an Eppendorf tube containing one milliliter of PBS (3.1.1.3). The leptospires were pelleted by centrifugation at 13,000 x g for 15 min at 4^0 C. Washed the pellet twice with sterile PBS and then resuspended in 25µl sterile triple glass distilled water. The samples in Eppendorf tubes were placed on a boiling water bath for 10 min and immediately kept on ice for 30 min. These were then centrifuged at 5000 x g for 10 min and the supernatant was used for PCR reaction.

3.1.2.1 .2b Reconstitution experiments with milk samples

Milk samples were seeded with reference strain of *L.pomona* in order to standardize the DNA extraction procedure from the milk samples.

A drop of culture of organism was inoculated into approximately 5ml of milk sample, mixed well and one millitre of this sample was transferred to an Eppendorf tube. Milk sample was centrifuged at 13,000 x g for 15 min at 4^0 C. After the centrifugation milk fat was found raised to the top and accumulated at the top of supernatant. Fat and supernatant was removed and washed the pellet twice in sterile PBS and then resuspended in 25µl sterile triple glass distilled water. The samples in Eppendorf tubes were placed on a boiling water bath for 10 min and immediately kept on ice for 30 min. These were then centrifuged at 5000 x g for 10 min and the supernatant was used for PCR reaction.

3.1.2.1.2c Reconstitution of Primers

Primers obtained in lyophilized form were reconstituted in sterile glass distilled water to a concentration of 200 pM. The tubes were kept at room temperature with occasional shaking for one hour. The tubes were spun to pellet down the insoluble particles, and the stock solution was distributed into 10 µl

aliquots and stored at -20 ^{0}C. At the time of use aliquots were thawed and further diluted ten fold to obtain a concentration of 20 pM/µl before using for PCR.

3.1.2.1.2d Genus specific PCR

The template DNA prepared from reference strain was subjected to genus specific PCR using primers A and B. The total volume of each reaction mixture was 25µl . A master mix was prepared to contain the following reagents

Reagents	Quantity
Triple distilled water	68 µl
Magnesium Chloride	2.5 µl
10x PCR buffer	12.5 µl
Primer A	5 µl
Primer B	5 µl
dNTPs	5 µl
Taq polymerase	2µl

Twenty microliter of master mix was distributed into five tubes. Negative control with triple distilled water was also set to monitor the contamination .The PCR was carried out in an automated thermal cycler (Mastercycler gradient, Eppendorf-Netheler-Hinz GmbH, Hamburg).

3.1.2.1.2e Programme of Amplification

The programme of amplification was as follows

	First cycle	**28 cycles**	**Final cycles**
Denaturation	94°C for 3 min	94°C for 1 min	94°C for1 min
Annealing	63°C for 1.5 min	63°C for 1.5 min	63°C for 1.5 min
Extension	72°C for 1 min	72°C for 1 min	72°C for 10 min

3.1.2.1.2f Detection of PCR Products

Submarine Agarose gel electrophoresis in one per cent agarose gel in 1x TAE buffer was performed for the detection of amplified products. To 250 mg agarose added 25 ml of 1x TAE buffer, dissolved by heating and cooled to 50°C. To this Ethidium bromide (3.1.1.5e) was added to a final concentration of 0.5 µg/ml.

The two edges of a clean, dry, gel platform were sealed with adhesive tape and the comb was kept in proper position before pouring agarose. Once the gel was set comb and adhesive tape were removed and was then placed in buffer tank. Ten microlitre of PCR product mixed with one microlitre of 6x gel-loading buffer was loaded into the wells. Five microlitre of DNA molecular size marker (3.1.1.5g) was also loaded in the last well.

3.1.2.1.2g Recording of results.

Gel was visualized under uv transilluminator (Hoefer, USA) and the results were documented on gel documentation system (Bio-Rad Laboratories,USA).

3.1.2.2 Detection of leptospires in mastitic milk

3.1.2.2.1 Preparation of template DNA from milk samples

One milliliter of milk sample was inoculated into three milliliter of Fletcher's semi solid medium. One milliliter of this diluted milk sample was transferred to an Eppendorf tube and centrifuged at 14000 x g for 10 min at 4° C, washed twice with PBS(3.1.1.3) and then resuspended in 15µl of sterile triple distilled water. Then, the samples were placed in boiling water bath for 10 min and immediately kept on ice for 30 min.Before doing the PCR the samples were thawed at room temperature and centrifuged at 5000 x g for 10 min to remove sediments .The supernatant was used as template for PCR.

Direct milk samples (without inoculating into medium) were also screened by PCR. In that case, initial quantity of sample taken was 15ml. Rest of the procedure was same as that of diluted sample.

3.1.2.2.2 Detection of leptospiral DNA

The template prepared from the milk samples were subjected to genus specific PCR as described for reference strain for the detection of leptospiral DNA using the primers A and B.

3. 2 ISOLATION OF *Leptospira*

3. 2.1 Sample collection

Isolation of *Leptospira* was mainly tried from mastitis milk, especially haemagalactia cases submitted to the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy. Milk samples were also collected from suspected cases presented to the nearby veterinary hospitals and from University Livestock Farm (ULF), Mannuthy.

3. 2.2 Glassware

Screw capped test tubes (Borosil brand) and steristoppered test tubes of (Reviera brand) were used in this study. The glassware were washed using mild soap solution, rinsed in running tap water and then in double glass distilled water, dried and sterilized in hot air oven at 160° C for 60 min. The caps were treated similarly and sterilized by autoclaving at 121° C for 15 min under 15 lbs pressure.

3. 2.3 Rabbit serum

The rabbit blood was collected by cardiac puncture into a sterile syringe containing one or two drops of distilled water and was allowed to clot at room temperature. It was then transferred to 4° C, kept overnight and the serum was separated.The serum was inactivated at 56° C for 30 min, filtered through seitz filter and stored at -20° C.

3. 2.4 5-Fluorouracil(5-FU) solution

Hundred milligrams of 5-FU was added to five millilitre of sterile triple distilled water. To this 0.1 to 0.2 ml of 0.1N NaOH was added and heated to dissolve. The pH was adjusted to 7.4 to 7.6 using 0.1N NaOH and the volume was made upto 10 ml with sterile triple distilled water. The solution was sterilized by filtration through a 0.2 μm membrane filter and stored at -20^0 C.

3. 2.5 Medium used for isolation

3. 2.5a Fletcher's Semisolid Medium

The medium was prepared by autoclaving 0.25g of the Fletcher *Leptospira* medium base (Hi - media) in 90 ml of triple distilled water at 121^0 C for 15 minutes under 15 lbs pressure, to which pooled, heat inactivated slightly haemolysed rabbit serum was added at 10 per cent level, after cooling the medium to 55-60^{0}C. The medium used in this study contained the following ingredients.

Ingredients	Grams/ liter
Peptone	0.3
Beef extract	0.2
Sodium Chloride	0.5
Agar	1.5

The medium was dispensed in three to five milliliter quantities in screw-capped tubes, checked for sterility by incubating at 37^0 C for 48h and 28^0 C for 5 days and was then stored at 4^0 C until use.

3. 2.5bFletcher's Semisolid Medium with 5-Fluorouracil (5-FU)

One milliliter of 5-FU solution (3.2.4) was added to 100 ml of Fletcher's semisolid medium to obtain a final concentration of 100 µg/ml of medium. The medium was dispensed in three to five milliliter quantities in screw-capped tubes, checked for sterility by incubating at 37^0 C for 48 h and 28^0 C for 7 days and was then stored at 4^0 C until use.

3. 2.6 Method of isolation

Screw capped tubes containing three to five milliliters of Fletcher's semisolid medium were inoculated with one or two drops of suspected milk samples, with aseptic precautions. The inoculum was thoroughly mixed with medium and one milliliter of medium was transferred to a second tube and from this to a third tube. All the inoculated tubes were incubated at 28-30^0 C for two to three months.

3.3 MATERIALS FOR THE DETECTION OF *Mycoplasma*

3.3.1 Sample collection

Milk samples were collected from suspected cases of *Mycoplasma* mastitis. This constituted samples from sub clinical cases also.

3.3.1.2 Reference strain of Mycoplasma bovis

Reference strain of *Mycoplasma bovis* was procured from Division of biologicals and standardisation ,IVRI, Izatnagar.

3.3.1.3 Buffers and Reagents for PCR

Buffers and Reagents used were same as those given under detection of leptospirosis except for primers and molecular size markers.

3.3.1.3a *Mycoplasma genus-specific primers MGSO and GPO3 (Marois et al., 2000)*

5' GGC AGC AAA CAG GAT TAG ATA CCC T3'

5' TGC ACC ATC TGT CAG TCT GTT AAC CTC3'

Primers were obtained as custom made 100 nM standard desalted oligonucleotides with OD-260 values of 17.2 and 18.1 respectively from IDT, Inc. USA.

3.3.1.3b *Mycoplasma bovis-specific primers PpMB 920-1 and PpMB 920-2 (Hotzel et al., 1993)*

5' GGC TCT CAT TAA GAA TGT C 3'

5' TTT TAG CTC TTT TTG AAC AAA T 3'

(standard desalted oligonucleotides IDT, Inc. USA).

3.3.1.3c DNA Molecular size Marker (330 microgram per milliliter)

a) pUC 19 DNA /*Msp* I Digest consisting of 9 double –stranded DNA segments of 34/ 34, 67, 111/110, 147, 242, 331, 404 and 501/489 bp.

b) pBR 322 DNA/*Alu* I Digest consisting of double – stranded DNA segments of 63/57/49, 100/90, 226, 257, 281, 403, 521, 659/656 and 908 bp.

c) 500 bp DNA Ladder consisting segments of 500,1000. 1500, 2000, 2500, 3000, 3500, 4000, 4500 and 5000

3.3.2 Methods

3.3.2.1 *Standardization of molecular techniques employing the reference strains*

3.3.2.2 Maintenance of reference strain

Mycoplasma bovis isolate was maintained by sub culturing in buffalo heart infusion broth. The purity of culture was assessed by culturing on blood agar

3.3.2.3 Polymerase Chain Reaction for the Detection of M.bovis

3.3.2.3a Preparation of template DNA

One milliliter of BHI broth culture of *M.bovis* was transferred to an Eppendorf tube.It was spun at 13,000 x g at 4° C for 20 min, supernatant discarded and the pellet was resuspended in PBS (pH 7.4) by agitating in a vortex mixer. The cell pellet was washed twice in PBS at 13,000 x g for 15 min at 4° C. Resuspended the pellet in a final volume of 20µl of PBS. The samples were placed on boiling water bath for 10 min and immediately kept on ice for 30 min. The lysate was thawed and centrifuged at 5000 x g for five minutes and supernatant was stored at –20° C for further use as template for PCR reactions..

3.3.2.3b Reconstitution experiments with milk samples

Milk samples were seeded with reference strain of *M.bovis* in order to standardize the template preparation from the milk samples.

A drop of culture of organism was inoculated into approximately 5ml of milk sample, mixed well and one millitre of this sample was transferred to an Eppendorf tube .Milk sample was centrifuged at 13,000 x g for 15 min at 4^0 C. During the centrifugation milk fat was raised to the top and found accumulated at the top of supernatant. After removing the fat and supernatant washed the pellet twice in sterile PBS and then resuspended in 25µl sterile triple glass distilled water. The samples in Eppendorf tubes were placed on a boiling water bath for 10

min and immediately kept on ice for 30 min. These were then centrifuged at 5000 x g for 10 min and the supernatant was used for PCR reaction.

3.3.2.3c Reconstitution *of Primers*

Same as that of *Leptospira* primers.

3.3.2 .3d *Mycoplasma genus-specific PCR*

The PCR reaction was carried out as per the method elaborated by Marois *et al.*, (2000) with modifications.

Polymerase chain reaction was performed in a total volume of 25-microlitre reaction mi xtures. A master mi x was prepared before setting up the PCR reaction by combining the following reagents in 25-microlitre volumes.

Reagents	Quantity
Triple distilled water	68µl
Magnesium Chloride	2.5µl
10 x PCR buffer	12.5µl
Forward primer, MGSO (20 pM/ml)	5µl
Reverse primer, GPO3 (20 pM/ ml)	5µl
dNTPs	5µl
Taq polymerase	2µl

Twenty microliter of master mi x was distributed into five tubes. Negative control with tripple distilled water also set to monitor the contamination .The PCR was carried out in an automated thermal cycler (Mastercycler gradient, Eppendorf-Netheler-Hinz GmbH, Hamburg).

3.3.2 .3e Programme of Amplification

Initial denaturation		90° C for 1 min
40 cycles	denaturation	95° C for 15 sec
	annealing	58° C for 20 sec
	elongation	75° C for 20 sec
One cycle of	denaturation	95^0C for 15 sec
	annealing	58° C for 45 sec
	extension	75° C for 5 min

3.3.2 .3f *Identification of PCR Product*

Procedure same as given in (3.1.2.1.2f)

3.3.2 .3g Recording *of Result*

Procedure same as given in (3.1.2.1.2g)

3.3.2 .3h *M.bovis* Species specific PCR

The template prepared from reference strain was subsequently subjected to *M.bovis* species specific PCR. A twenty five µl reaction mixture containing the following reagents were prepared.

10 X PCR assay buffer 2.5 µl

$MgCl_2$ 0.5 µl

Forward primer, PpMB 920-1(20 pM/ml) 1 µl

Reverse primer, PpMB 920-2 (20 pM/ml) 1 µl

dNTPs (200 µM) 1 µl

Taq DNA polymerase 1 unit

Template DNA 5 µl

Triple distilled water 13µl

3.3.2 .3i Programme of Amplification

Initial denaturation		90° C for 2 min
30 cycles	denaturation	94° C for 30 sec
	annealing	48° C for 60 sec
	elongation	72° C for 2.5 min
Final e xtension		72° C for 5 min

3.3.2 .3j *Identification of PCR Product*

Procedure was same as that of (3.1.2.1.2f)

3.3.2 .3k Recording *of Result*

The gel was visualized under UV trans-illuminator (Hoefer, USA) and the results were documented on gel documentation system.

3.3.2.4 Detection *of Mycoplasma* in mastitic milk samples

3.3.2.4a Preparation of template DNA from milk samples

One milliliter of milk sample was inoculated into three milliliter of buffalo heart infusion broth . These broth media were incubated till a colour change from pink to yellow was visible. About one milliliter of the broth was

spun at 13,000 x g at 4° C for 20 min. The supernatant was then discarded and the pellet was resuspended in PBS (3.1.1.3) of pH of 7.4 by agitating in a vortex mixer. The cell pellet was washed twice in PBS at 13,000 x g for 15 minutes at 4° C. Resuspended the pellet in a final volume of 20 microlitre of PBS. Samples were kept in boiling water bath at 100° C for 10 min and immediately placed on ice for at least 30 min. Lysate was then centrifuged at 5000 x g for 5 min to remove the debris. Supernatant containing the DNA was collected and stored at – 70° C till use.

Direct milk samples (without inoculating into media) were also screened by PCR. In that case initial quantity of sample taken was 15 ml. Rest of the procedure was same as that of broth enriched sample.

3.3.2.4b Detection of Mycoplasmal DNA

The templates prepared from the milk samples were subjected to genus specific PCR as described for reference strain for the detection of *Mycoplasma* DNA using the primers MGSO and GPO3 (Maoris *et al.*, 2000). Sample found Positive was also subjected to species specific PCR using the *M bovis* primers PpMB 920-1 and PpMB 920-2(Hotzel *et al.*, 1993)

3.4 ISOLATION OF *Mycoplasma*

3.4.1 Glassware

Glassware of Borosil and Riviera brands were used in this study. The glassware were washed using mild soap solution, rinsed in running tap water and then in double glass distilled water, dried and sterilized in hot air oven at 160° C for 60 min. The caps were treated similarly and sterilized by autoclaving at 121° C for 15 min under 15 lbs pressure.

3.4.2 Media Ingredients

3.4.2a *Buffalo Heart Infusion*

Buffalo heart infusion was prepared by boiling 500 g minced fat free buffalo heart muscle with 1000 ml distilled water for 45 minutes. It was filtered through a muslin cloth with cotton pad, distributed in small quantities, sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure and stored at -70° C until use.

3.4.2b *Horse Serum*

Blood was obtained observing aseptic precautions from horses maintained by 1 (K) R&V Sqr., KAU Unit, Mannuthy. Serum was separated and sterilized by passing through seitz filter and stored at -70° C in 20 ml aliquots till use.

3.4.2c *Yeast E xtract*

Baker's yeast 250 g was suspended in one litre of distilled water, heated to boiling point, cooled and centrifuged for 20 minutes at 3000 x g. The supernatant fluid was decanted and adjusted to pH 8.0 with 0.1 M NaOH. This was clarified by filtration through Whatman No.1 filter paper and then sterilized by passing through seitz filter. The extract was stored in small aliquots of 10 ml at –70° C.

3.4.2d *Glucose Solution*

Fifty per cent of glucose stock solution was prepared in distilled water, sterilized by filtration and stored at -70 ° C in 10 ml aliquots.

3.4.2e *Antibiotics*

Penicillin G (10, 0000 1U/5ml) prepared in sterile distilled water and stored at -70 °C in 10 ml aliquots.

3.4.2f *Thallium Acetate*

Thallium acetate solution (2.5 per cent) was prepared in sterile distilled water and stored at –70°C in 10 ml aliquots.

3.4.2g *Phenol Red*

Phenol red 0.1g was ground in 2.8 ml of 0.1 M NaOH, and then made upto 100 ml in sterile distilled water, sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure and stored at 4°C.

3.4.2h *Deo xyribo Nucleic Acid*

Deoxyribo nucleic acid 0.2 per cent solution was prepared in distilled water, sterilized by filtration and stored at -70 ° C in 10 ml aliquots.

3.5 MEDIUM USED FOR ISOLATION

3.5a Buffalo Heart Infusion Broth (BHI Broth)

Buffalo heart infusion	-	700 ml
Bactopeptone (Difco)	-	11 g
Phenol red	-	10 ml
NaCl	-	5 g
Glucose	-	2 ml
Horse serum	-	200 ml
Yeast e xtract	-	20 ml
Thallium acetate	-	20 ml
Penicillin	-	10 ml
Deo xyribo nucleic acid	-	1ml

Bactopeptone was dissolved in buffalo heart infusion, sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure and supplemented with the

remaining ingredients under aseptic conditions. The pH was adjusted to 7.8. About two to three millilitres of BHI broth were dispensed into screw-capped glass tubes of five millilitres capacity, kept for sterility checking at 37° C for 48 hours and after that stored at 4° C to be used within 14 days.

3.5 b *Buffalo heart Infusion agar (BHI Agar)*

PPLO agar base (Difco) - 700 ml

Prepared by dissolving 35 g of the agar base in 700 ml distilled water and sterilized by autoclaving at 121° C for 15 min at 15lbs pressure.

Buffalo heart infusion - 50 ml

Horse serum - 150 ml

Yeast e xtract - 100 ml

Glucose - 20 ml

Penicillin - 10 ml

Thallium acetate - 20 ml

Deoxyribo nucleic acid - 1ml

pH adjusted to 7.8.

Mycoplasma agar base was allowed to cool to 45 to 50° C and supplemented with the remaining ingredients under aseptic conditions. Agar was then poured into 55 mm diameter Petri dishes at a depth of five to si x mm, kept for sterility checking at 37° C for 48 hours and stored at 4° C to be used within 14 days.

3.6 CHARACTERIZATION OF MYCOPLASMA ISOLATES

3.6a *Diene's Stain*

Methylene blue 2.5 g

Azur II	1.25 g
Maltose	10 g
Sodium carbonate	0.25 g
Distilled water	100 ml

Sterilized by filtration and stored at room temperature

3.6 b *Diene's Staining Technique*

Agar plates with *Mycoplasma* colonies were flooded with Diene's stain diluted 1:100 in normal saline, for one minute. E xcess of stain was removed and incubated at 37° C for 30 to 60 min. Mycoplasma colonies stain an intense blue at the centre with light blue periphery. Colonies of *Mycoplasma* were e xamined for the characteristic fried egg appearance, colour, te xture, size and for characteristic staining with Diene's stain.

3.7 STANDARDIZATION OF MEDIA EMPLOYING THE REFERENCE STRAIN

Reference strain of *Mycoplasma bovis* was procured in the freeze dried form from the Division of biological and standardizations ,IVRI, Izatnagar. Initially, it was reconstituted in BHI broth and mixed well . Two drops from this broth were aseptically transferred to a fresh broth and also to a BHI agar plate respectively. Inoculated plates and broths were incubated at 37°C in a candle jar. Colonied developed on solid mediun were identified by Diene's staining.

3.8 METHOD OF ISOLATION

3.8a Sample Collection

Milk samples were mainly collected from the clinical and sub clinical mastitis cases presented to Veterinary Hospital, Mannuthy, from nearby farms, from samples submitted to microbiology lab, College of Veterinary and Animal Sciences, Mannuthy and also from ULF, Mannuthy. Milk samples were collected after screening by CMT. Samples collected include individual cow sample, bulk

milk sample and cow composite sample. After collection samples were immediately transported over ice to the laboratory.

3.8b Processing of Samples

Immediately after collection, milk samples were inoculated into BHI broth. 500µl of sample was inoculated into two milliliter of broth medium. After mixing properly aseptically transferred one milliliter of broth to second tube and from this to third tube. All the inoculated broths were transferred without delay to a candle jar and incubated at 37° C. The caps of the broth medium containers were tightly closed before incubation so as to avoid spurious changes in pH.

Broth media that have turned to yellow were plated to BHI agar and incubated at 37° C in a candle jar. The DNA template was prepared from this broth and the *Mycoplasma* genus-specific PCR was performed.

Some of the milk samples were also directly streaked on BHI agar. Agar plates (directly streaked with samples or those subcultured from the broth) were examined daily under 10 x objective of the microscope for the presence of mycoplasmal colonies. All the negative plates were kept incubated upto 21 days whereas broth were incubated till an appreciable colour change of the broth from pink to orange or yellow was evidenced or upto 21 days whichever was earlier.

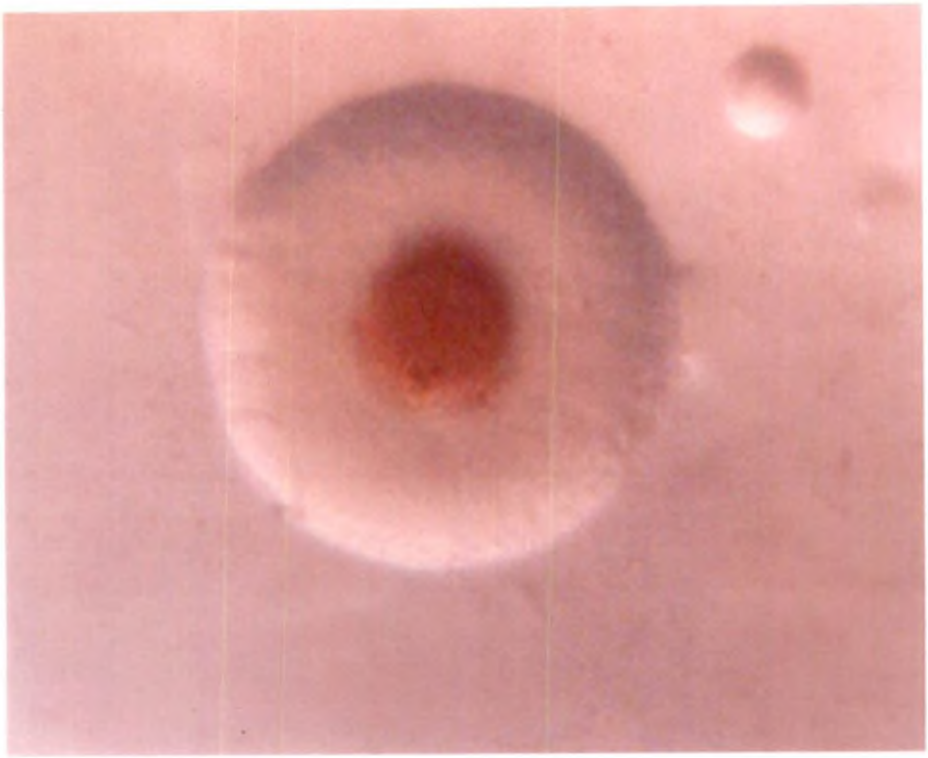

Fig. 1 ***Mycoplasma bovis*- Single Colony (10 x Magnification)**

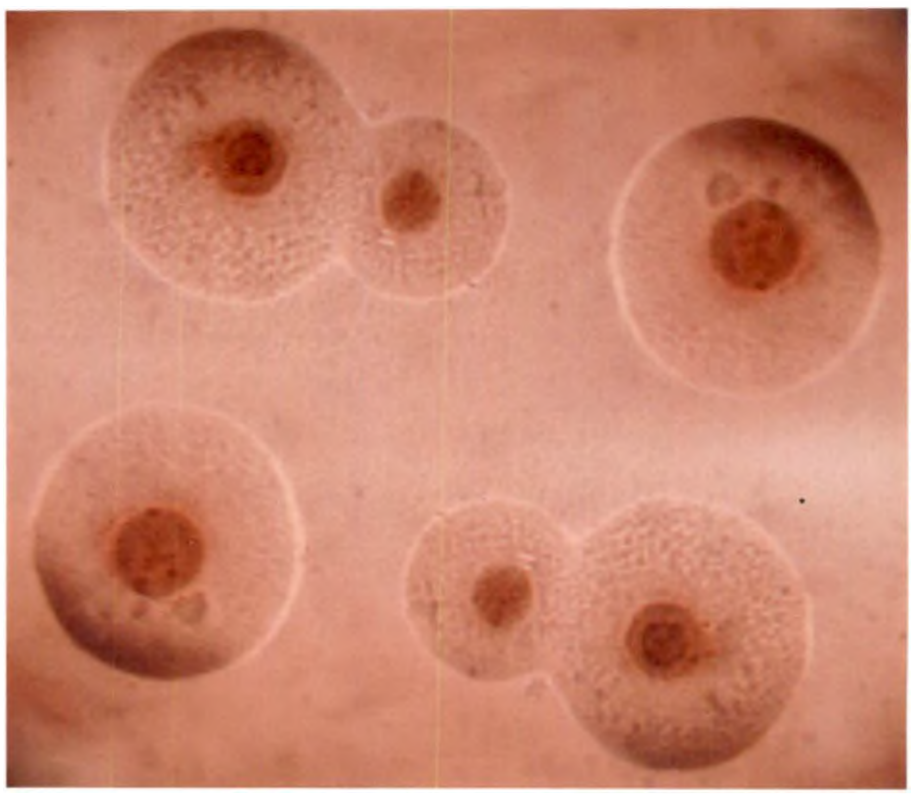

Fig. 2 Colonies of *Mycoplasma bovis* showing fried - egg apperance (10 x Magnification)

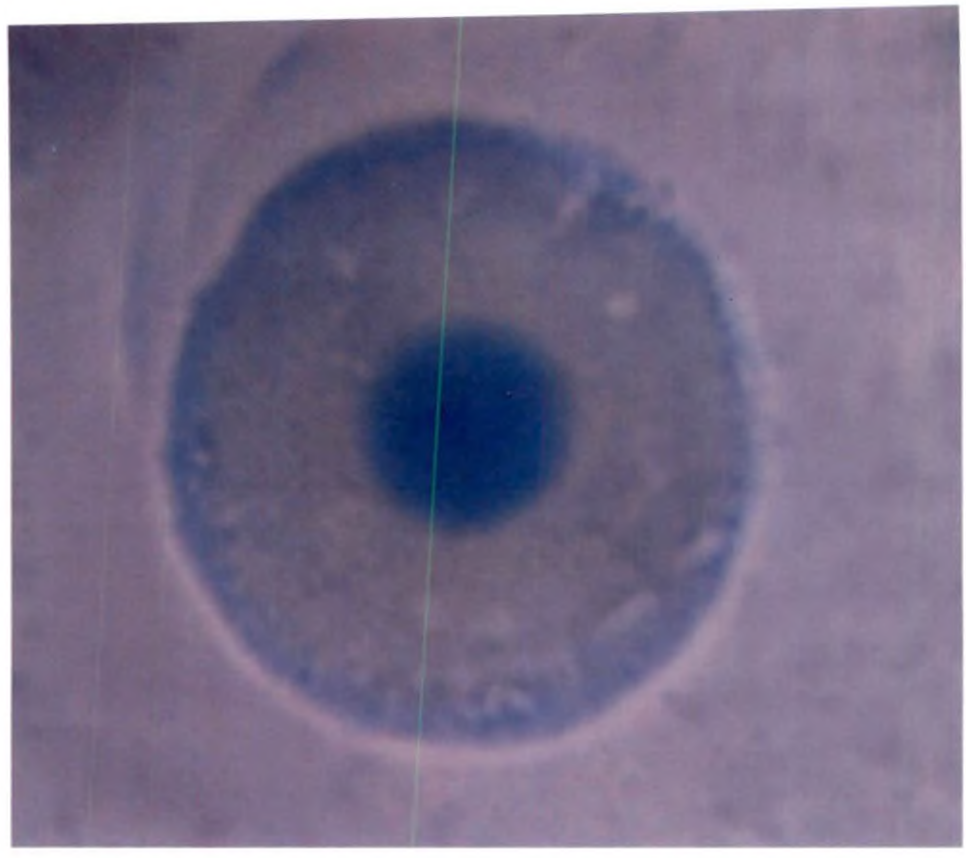

Fig. 3 *Mycoplasma bovis*- Single Colony stained by Diene's method of staining (10 x Magnification)

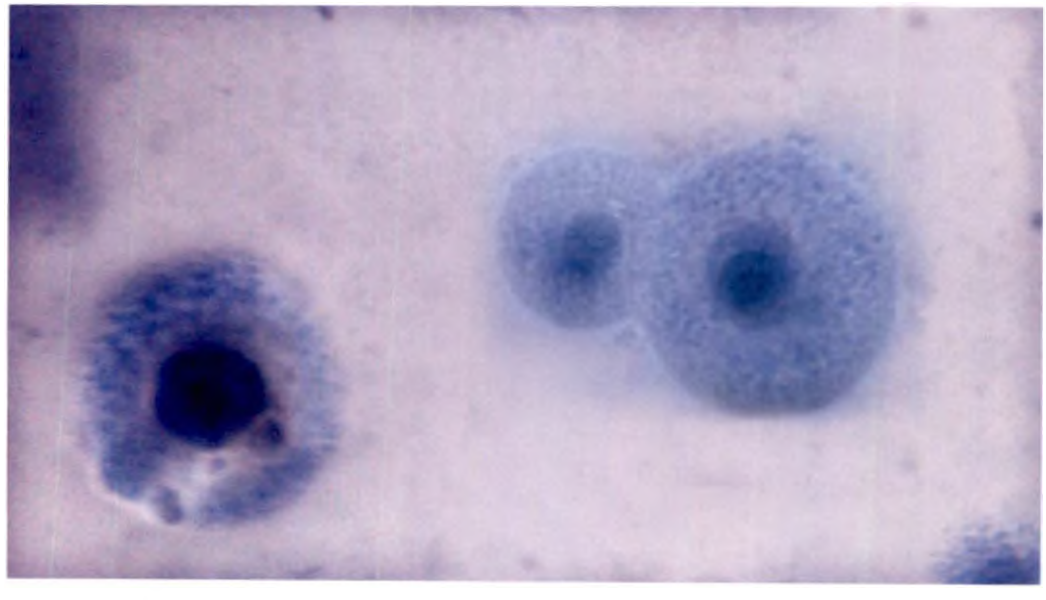

Fig. 4 *Mycoplasma bovis*- Colonies stained by Diene's method of staining (10 x Magnification)

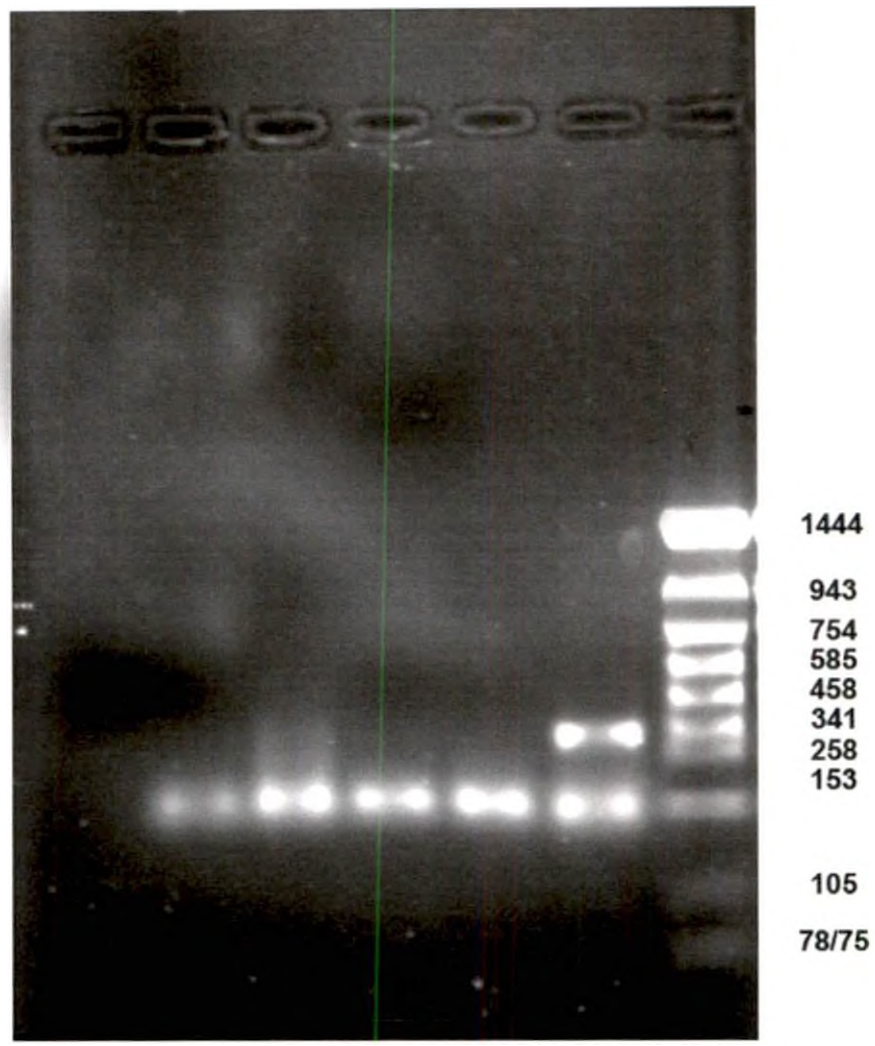

Fig. 5. Agarose gel (1per cent) electrophoresis of *Leptospira* genus-specific PCR product

Lane 1: Negative control
Lane 2,3,4,5: Negative milk sample

Lane 6: *Leptospira* reference strain
Lane 7: puc 18/*sau 3* A1- puc 18/ *Taq* 1 Diges

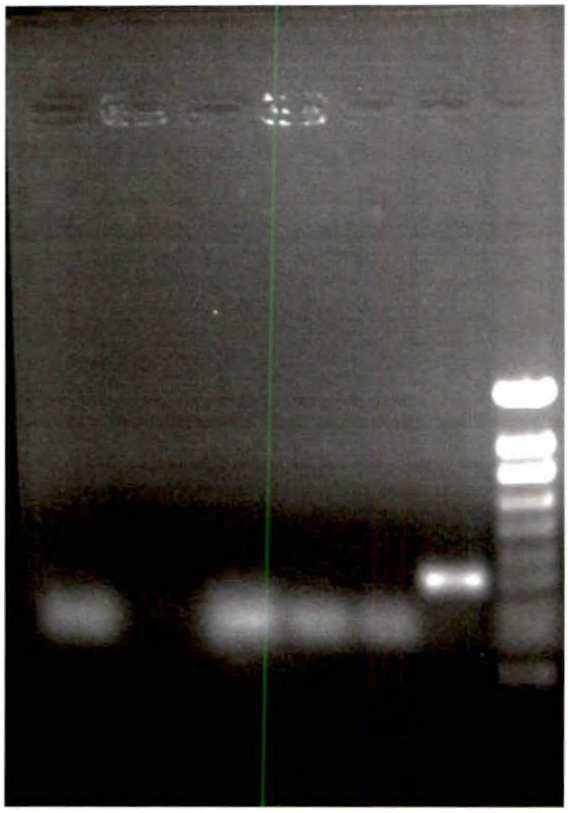

Fig. 6. Agarose gel (1per cent) electrophoresis of *Mycoplasma* genus-specific PCR product

Lane 1,3,4,5: Negative milk sample
Lane 2: Negative control
Lane 6: *Mycoplasma* reference strain
Lane 7: puc 18/*sau 3* A1- puc 18/ *Taq* 1 Digest

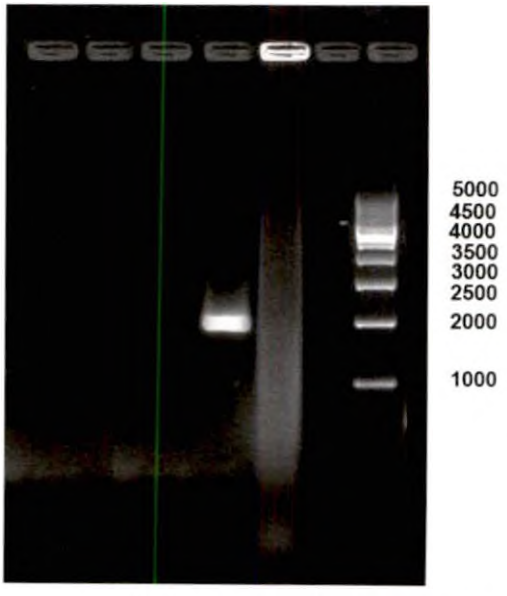

Fig. 7. Agarose gel (0.8 per cent) electrophoresis of *Mycoplasma bovis* species-specific PCR product

Lane 1,2,3,5: Negative milk sample
Lane 6: Negative control
Lane 4: *Mycoplasma* bovis reference strain
Lane 7: 500 bp DNA Ladder

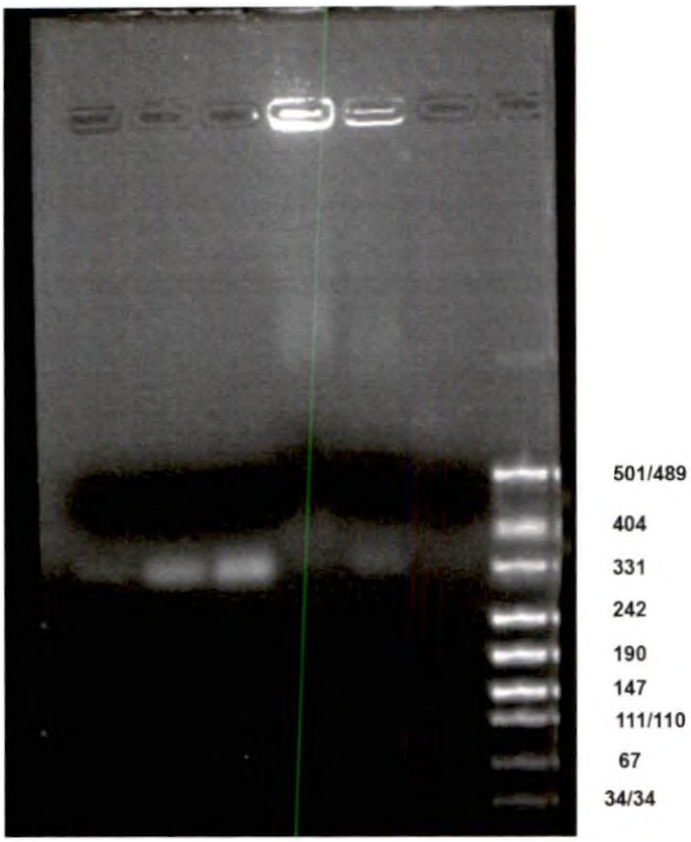

Fig. 8. Agarose gel (1per cent) electrophoresis of Leptospira genus-specific PCR product of representative milk sample

Lane 2: Postive milk sample
Lane 1: Negative control

Lane 3: *Leptospira* postive control
Lane 4,5,6: Negative sample
Lane 7: puc 9/*Msp 1* Digest

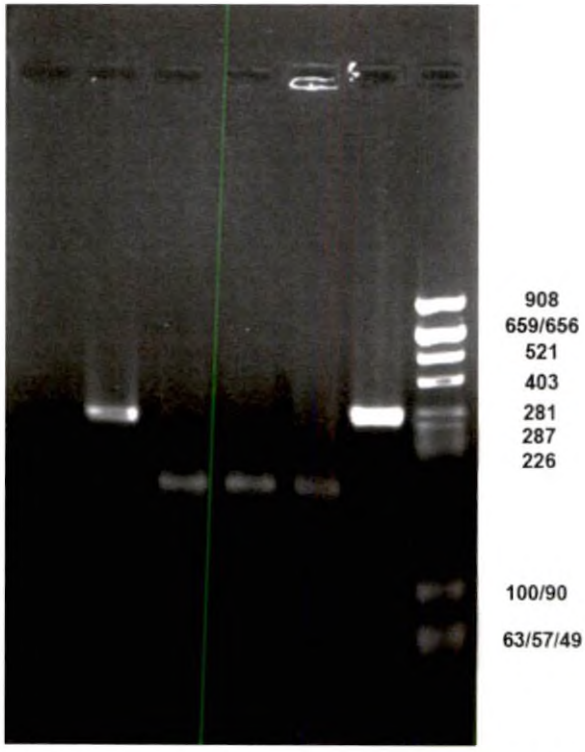

Fig. 9. Agarose gel (1per cent) electrophoresis of *Mycoplasma* genus-specific PCR product of representative milk sample

Lane 2: Postive milk sample
Lane 1: Negative control

Lane 3,4,5: Negative sample
Lane 6: *Mycoplasma* postive control
Lane 7: pBR322 DNA/ *Alu* 1 Digest

4. RESULTS

4.1 STANDARDIZATION OF MEDIA EMPLOYING REFERENCE STRAIN

4.1.1 *Leptospira*

Fletcher's semi solid media enriched with 10 per cent rabbit serum dispensed in screw capped test tubes as well as steristoppered test tubes could support the growth of *Leptospira*. Hence it was used f or maintenance and routine subculturing of the reference strain of *Leptospira pomona*. Cultures were incubated at 28°C in BOD incubator. Slow growth of organism in an enriched media predisposes the cultures to contamination. In order to avoid the contamination and to purify the cultures selective inhibitor like 5-FU (pyramidine analogue) was added at a concentration of 100µg per ml. Cultures could not be maintained more than two months in screw capped test tubes due to loss of moisture by evaporation. This necessitated the routine sub culturing of organism at one month interval. The growth of organism was evidenced by the formation of sub surface ring called Dinger's ring, indicating the microaerophilic nature of organism. It was developed in 7 to 10 day old culture. To assess the purity of culture one or two drops of medium was observed under DFM after one week of culture. The characteristic serpentine motility of organism helped to differentiate it from coccobacilli and bacilli which were usual contaminants observed in the media. Contamination of cultures was a problem in the humid environment prevalent in the rainy season. Motility was observed up to three months in cultures maintained in screw capped test tubes.

4.1.2 *Mycoplasma*

Reference strain of *Mycoplasma* procured in freeze dried form could be successfully revived in the BHI broth and BHI agar. Since the organism required CO_2 tension the cultures were incubated at 37°C in a candle jar. The growth of

organism as evidenced by a change in the colour of broth medium from pink to yellow, was observed within 24 h of incubation , in solid medium the colonies developed very slowly, only on fourth day of incubation. But the solid medium plated with broth culture produced the colonies within 48 h . *Mollicute* colonies are small and difficult to visualize with unaided eyes. Hence plate media were examined with the aid of light microscope, when colonies with typical fried egg appearance was observed. This Umbonate appearance is the result of central portion of the colony embedding into the agar with a peripheral zone of surface growth. Colonies with typical umbonate morphology were stained directly with the Diene's stain. *Mycoplasma* colonies stained an intense blue at the centre, with light blue periphery.

4.2 STANDARDIZATION OF GENUS SPECIFIC PCR EMPLOYING REFERENCE STRAIN

4.2.1 Leptospira

In order to assess the specificity of primers A and B they were used to amplify the template DNA prepared from other organisms like *E. coli* and St*aphylococcus* .Primers didn't produced any cross amplification with other bacterial isolates. So the primer pair A and B were selected in this study to detect the leptospiral DNA in clinical samples.

The template DNA produced from reference strain of Leptospira was subjected to genus specific PCR, along with negative control. Polymerase chain reaction produced an amplicon of 331bp which was observed by analysing the electrophoresed gel under UV transillumination. In negative control, amplification product was not detected.

4.2.2 Mycoplasma

The primer pair GPO_3 and MGSO were found to be specific for *Mycoplasma*, as they did not produce any cross amplification of other bacterial DNA. Hence the primer pair were selected for the amplification of *Mycoplasma* DNA from all clinical samples. An amplicon of 270 bp produced was detected by analysing the electrophorsed gel under UV trans illumination. In negative control, no amplicon was detected.

4.3 SPECIES DIFFERENTIATION OF REFERENCE STRAIN OF MYCOPLASMA

The primer pair PpMB 920-1 and PpMB 920-2 when used to amplify the DNA prepared from *E. coli, Staphylococcus* and *Leptospira pomona* did not evidence any amplification, indicating the specificity of primers .Hence this primer pair was selected for the amplification of genome of *M.bovis* from clinical samples.

Polymerase chain reaction was carried out using the DNA prepared from reference strain of *Mycoplasma.* A negative control was also set along with positive strain to monitor the contamination. Production of an amplicon of 2kb which was observed under UV tansillumination of electrophorosed gel confirmed the organism as *M. bovis.*

4.4 DETECTION OF LEPTOSPIRA AND MYCOPLASMA IN MILK SAMPLES

4.4.1 Leptospira

Milk samples suspected for leptospirosis were subjected to genus specific PCR. The PCR failed to amplify the DNA from direct milk sample.Hence initially the milk samples were inoculated into Fletcher's semi solid media.Genus

specific PCR was performed with this diluted milk sample and was incubated at 28° C in BOD incubator. Out of fifty samples screened only one sample produced a positive result with an amplicon of 331 bp. It was a typical case of haemagalactia and the animal exhibited clinical signs suggestive *of* leptospirosis. No other bacteria could be isolated from this sample.

4.4.2 Mycoplasma

As in the case of milk sample suspected for leptospirosis, direct screening by PCR of milk sample from suspected mycoplasmal mastitis cases failed to produce any amplification of DNA. Inhibitory substances present in the milk interfered with the movement of DNA.To overcome this difficulty, initially the samples were inoculated into the BHI broth and incubated until the colour change was evident. Template was prepared from this broth media and genus specific PCR was carried out using these templates. Out of the fifty samples screened only one sample produced a positive result. The size of amplicon produced was 270 bp as compared with molecular markers.Polymerase chain reaction using species specific primers PpMB 920-1 and PpMB 920-2 did not produce any amplified product.

4.5 ISOLATION OF LEPTOSPIRA AND MYCOPLASMA FROM MILK SAMPLES

4.5.1 Leptospira

All the haemagalactia cases with the history and clinical signs simulating leptospirosis were used for isolation .Milk was aseptically transferred to the Fletcher's semi solid medium using a freshly drawn pasture pipette .After inoculation the samples were incubated in a BOD incubator at 28°C. Cultures were examined for growth every weekly intervals under DFM. But the organism could not be isolated from any of the samples including the sample from which the leptospiral DNA was detected.

4.5.2 Mycoplasma

Milk for isolation of *Mycoplasma* was collected from both clinical and sub clinical cases of mastitis. Besides the individual sample both composite sample and bulk tank milk were collected from large herds after screening by CMT. Samples were transported to lab over ice without delay. Milk samples were transferred aseptically into BHI broth and incubated at 37°C in candle jar. Broth in which colour change was evident was carefully plateted to solid media. All the plates were incubated at 37°C in candle jar upto 21 days. But *Mycoplasma* could not be isolated from any of the milk samples.

Discussion

5. DISCUSSION

5.1 LEPTOSPIROSIS

Since the 1950s, many case reports have associated abortion, weak and still born calves, infertility, haemolytic anaemia and mastitis with leptospiral infection (Miller, *et al.*, 1991).

Susceptible hosts can become infected through mucous membranes or abraded skin. After 4 to 10 days, the host becomes bacteraemic, this period lasting from hours to 7 days and is characterized by pyrexia, presence of leptospires in the milk and anorexia. During the bacteraemia, leptospires will invade internal organs and the extent of the damage will depend on susceptibility of the host and virulence of organism (Thiermann, 1984).

The predominant manifestation of bovine leptospirosis is abortion. *L. hardjo* (host adapted serovar) are commonly isolated from cases of abortion. Two clinical conditions have been reported in cattle infected with serovar *hardjo*. Firstly, a sudden onset of agalactia, which is associated with acute bacteraemia and secondly abortion, which occurs as a chronic sequelae to infection (Ellis *et al.*, 1985).

Diagnosis of leptospirosis is usually based on the demonstration of antibodies with serological tests like MAT or ELISA. But active bovine leptospiral infection often occurs in the absence of detectable agglutinin titres. In chronic infection, serology does not provide reliable information on the carrier or shedding state (Ellis *et al.*, 1982).

Since cattle infected with leptospires fail to produce detectable antibodies an accurate diagnosis of infection requires direct demonstration of organism in tissues and other biomaterials. This is achieved by bacteriological culture (Zuerner *et al.*, 1988) but it is laborious and time consuming.

With the introduction of PCR, rapid detection of small numbers of leptospires in clinical sample has become possible due to specific amplification of leptospiral DNA. For epidemiological studies the assay used should be sensitive and allow many samples to be processed simultaneously. The specificity of the assay can be adjusted by the choice of primers (Van Eys *et al.*, 1989).

The present study was aimed to detect the leptospires in bovine mastitis cases using genus specific PCR. The method was standardised by employing standard reference strain. Attempts were also made to isolate the organisms from milk samples.

5.2 DETECTION OF LEPTOSPIRES

5.2.1 Polymerase Chain Reaction

The genus specific primers A and B designed by (Merien *et al.*, 1992) could amplify DNA from the reference strain. This shows that this primer is suitable to detect leptospires in clinical samples.

Merien *et al.* (1992) designed the primers A and B based on the sequence of 16S rRNA for the detection of leptospires in body fluid.

PCR has been accepted as a rapid and sensitive technique to detect the leptospires in the body fluids (Van Eys *et al.*, 1989: Merien *et al.*, 1992).

Brown *et al.* (1995) opined that PCR was more sensitive than culture for the detection of leptospires in the sera and urine samples from patients with severe leptospirosis.

Senthilkumar *et al.* (2001) proved that PCR assay can be used not only for blood and urine, but also for the samples like milk and CSF.

In the present study, out of the fifty pre enriched milk samples screened by PCR, only one sample was found positive. But the same sample was culture negative, indicating PCR was a sensitive test to detect leptospiral DNA in milk samples. This result is in accordance with the observations of Heinemann *et al.*(1999) who also detected leptospires in bovine semen samples using the primers A and B. But at the same time these samples were negative by culture.

The failure to get a positive result by culture could be due to the fact that the culture detects only the presence of viable organism. At the same time PCR could detect specific target DNA , irrespective of whether it is from viable organism or not. Hence the detection of leptospiral DNA in the mastitis milk sample confirms the cause of mastitis.

Senthilkumar *et al.* (2001) could detect amplification of the leptospiral DNA in 50 per cent of milk samples screened by genus specific PCR. The presence of organism or its nucleic acid in the mastitis milk samples could confirm the cause of mastitis.

Sensitivity of PCR-based assays tends to be superior to bacterial culture, allowing the detection of small numbers of microorganism. Moreover, PCR provides a promising option for the rapid identification of bacteria (Cramonesi, 2006).

A PCR negative result does not eliminate the possibility of leptospiral infection as most of the samples reach in the laboratory after a heavy antibiotic therapy. Merien *et al.* (1992) have observed negative PCR results in two clinically and serologically positive cases of leptospirosis. In both these cases samples were collected late in infection (day 14 after the onset of the infection) and after heavy antibiotic treatment.

Brown *et al.* (1995) also found that PCR failed to detect leptospiral DNA in some samples. This might be due to the inhibitory factors present in the

samples that impeded amplification process, as well as when the leptospires were present in very low numbers , ie., below the detection level of 1-10 organism per ml.

5.3 ISOLATION OF LEPTOSPIRES

Ellis *et al.* (1976) first successfully isolated leptospires belonging to the Hebdomadis serogroup from milk and blood of cows with clinical mastitis. Among the 4 media used for isolation or organism, only EMJH found to support the primary route.

Higgins *et al.* (1980) could successfully isolate serotype Hardjo from milk and urine of dairy cows in which a sudden onset of agalactia was noticed

Adinarayanan and James (1980) succeeded in isolating leptospires in Stuart's or Fletcher's medium with sterile 10 percent haemoglobinised rabbit serum.

Theirmann (1981) attempted to isolate the leptospires from the milk using both solid and semisolid medium of BAP-80 and also by hamster inoculation. Solid BAP-80 was the only medium capable of isolating serovars hardjo and Szwajizak from cow's milk. But he could isolate the organism only during the first ten days of clinical mastitis and before the appearance of milk agglutinins.

Soman (2004) tried isolation of leptospires in Fletcher's or EMJH semisolid medium with 5-Fu and made primary isolation of leptospira in Fletcher's semisolid medium.

In the present study, Fletcher's semisolid medium was used for inoculating milk sample, since the reference strain has been maintained in the laboratory in this medium. But all the attempts to recover the organism from

suspected milk samples were unsuccessful. The PCR positive sample was also culture negative.

Stalheim (1965) had reported a leptospiral lysis by lipids from renal tissues and milk.

The recovery of leptospires from blood, urine, milk and tissues is often difficult because of the rapidity with which they die in the specimens after collection and its transient appearance in various tissues (Blood *et al.*, 1970).

Davidson (1980) opined that it was possible that the Unsaturated fatty acid (UFA) in milk accounted for the low culture success of leptospires using semisolid media.

Stalheim (1996) reported lipids present in the cow's milk have a antileptospiral activity which accounts for the failure of leptospires to be transmitted by milk.

Moreover according to Lefebvre (2004) milk was destructive to leptospires and not a promising source for culture.

Hence as per the observations made by the earlier workers antileptospiral factors present in the milk might have been responsible for failure to isolate the organism from the milk.. Eventhough the milk was diluted in the medium, traces of these factors present in the milk might have interfered with the growth of the organism.

5.4 MYCOPLASMOSIS

Mycoplasma has emerged as an important pathogen of bovine mastitis during the past 35 years. Though, several reports pertaining to the outbreak of bovine mycoplasmal mastitis are available from other parts of the world. Only limited reports are available from India. In India Pal *et al.*, (1982) isolated *M.*

bovigenitalium and *A. laidlawii* and Kaur and Garg (2000) isolated *M. bovis, M.bovirhinis* and *M. Canadense* from mastitis as well as non-mastitic milk samples.

Mastitis caused by *Mycoplasma* is refractory to most of the antibiotics. During the early latent state, treatment is effective in many cases. Best method to identify this group of pathogen is the direct culture on *Mycoplasma* media. However limitations with this culture procedure are the duration of culture and special conditions required for growth like enriched media and CO_2 tension (Nicholas, 1998).

Even though the serological test offers 90% specificity it is not generally used in the diagnosis of mycoplasmal infection due to cross reaction. Moreover, it requires the demonstration of increasing antibody titers that are reached only 10-14 days after the onset of clinical symptoms (Ayling *et al.*, 1997).

With the use of polymerase chain reaction (PCR) the chance of the detection of the organism is increasing in case of both early and chronic infections, compared to culture and serological methods (Hirose, *et al.*, 2001).

The present study was aimed to detect the mycoplasmas in bovine mastitis cases using genus specific PCR. The method was standardised by employing standard reference strain. Attempts were also made to isolate the organisms from milk samples.

5.5 DETECTION OF *Mycoplasma*

5.5.1 Polymerase Chain Reaction

Jasper (1981) opined that Mycoplasma infection might or might not produce an inflammatory reaction. Thus SCC would not accurately detect all

udders infected with *Mycoplasma.* Hence any control programme of *Mycoplasma* infection should use PCR to detect infected animal.

Ayling (1997) suggested that only small numbers of organisms are required for rapid identification of *Mycoplasma* isolates by using PCR.

In this study the genus specific primers MGSO and GPO3 (Marois *et al.*, 2000) could amplify DNA from the reference strain. This showed that these primers wer suitable to detect mycoplasmas in milk samples.

In the present study, all the fifty milk samples were subjected to PCR after enrichment in BHI broth. According to Kobayashi (1998) PCR failed to amplify the mycoplasmal DNA in direct milk sample but broth culture of same sample yielded an amplified product.

Out of fifty samples screened in this study, only one bulk milk sample yielded an amplified product. The genus specific PCR detected mycoplasmal DNA from one bulk tank milk collected from University Livestock Farm, Mannuthy in which increased SCC was evident. Positive sample yielded a band of 270 bp.

Ghadersohi *et al.* (1999) detected *M.bovis* on over 50% of bulk samples with SCC > $250x10^3$ cells/ml. The chronic high SCC count on affected herd shows that this agent play important role in aetiology of sub clinical and chronic mastitis.

Jasper (1981) found that some animals intermittently shedding *M.bovis.* This finding indicates that to detect animals infected with *M.bovis* frequent testing would be required.

Hayman and Hirst (2003) observed that the sensitivity of detection of *Mycoplasma* DNA by PCR depended upon the preparation of template DNA. Cleaner templates that contained fewer inhibitors were required.

Species specific primers PpMB 920-1 and PpMB 920-2 designed by Hotzel *et al.*, were used in this study to detect the presence of *M. bovis*. Even though these primers produced an amplicon of 2 kb for the reference strain (*M. bovis)* used, they failed amplify the template DNA prepared from sample found positive by genus specific PCR. This indicated that the causative agent for mastitis in this particular case could be another species of *Mycoplasma* and not *M. bovis*.

5.6 ISOLATION OF *Mycoplasma*

5.6.1 Standardization of Media Employing Reference Strain

In the present study, the media used were Buffalo heart infusion broth (BHI broth) and BHI agar which contained a rich protein base (e.g. heart infusion), cholesterol (horse serum), yeast extract, glucose and selective agents (ampicillin and thallous acetate).

Exogenous cholesterol was essential for the growth of *Mycoplasma* (Smith and Henrikson., 1996).

George (2003) opined that beef heart infusion supplemented some of the unspecified nutrients required for the growth of mycoplasmas.

5.6.2 Colony Characters of Reference Organism

Mycoplasma colonies appeared on BHI agar only on the fifth day of incubation at 37° C in a candle jar. But rapid development of colonies within 48 h noticed on BHIA plates streaked with broth culture. This result is in agreement with the observations of Gonzalez *et al.* (1995) who found that *M.bovis* was only

detected on agar plates where the samples were subcultured from broth and not on the direct plates.

The colonies obtained with *Mycoplasma* reference strain showed in general typical fried egg appearance. Apart from typical colonies, elongate and coalesced colonies were also obtained. According to (Yoder and Hofstad, 1964) *Mycoplasma* colonies frequently occured in ridges along the streak line, since closely adjacent colonies readily coalesce

The fried egg appearance of the *Mycoplasma* colonies had been well documented (Markham and Wong, 1952; Alder *et al.*, 1954; Frey *et al.*, 1968; Jain *et al.*, 1971; Power and Jordan, 1976).

5.6.3 Diene's Staining

In the present study, reference strain grown on BHI agar was identified as *Mycoplasma* based on the morphology. *Mycoplasma* colonies produced typical fried egg appearance on the agar surface and were confirmed by the Diene's method of staining. (Madoff, 1960)

The isolation of *M bovis* from milk sample is the definitive confirmation of *M.bovis* mastitis. The sensitivity of these culture based methods of detection depended on the continuous, as distinct from intermittent excretion of *M.bovis* from any infected quarter, the aseptic collection of milk from each quarter and the survival of *Mycoplasma* under the conditions of transit in sufficient numbers to be detected (Byrene *et al.*, 2000).

In the present study, samples collected included not only individual cow samples but also bulk tank sample and composite sample. But all the attempts to isolate the organism were unsuccessful. The organism could not be recovered even from PCR positive sample.

The presence of *Mycoplasma* in bulk tank milk was the result of shedding from infected quarters, even though a negative *Mycoplasma* culture from a bulk tank did not guarantee the absence of *Mycoplasma* mastitis in a herd. (Jasper *et al.*, 1966).

Weger *et al.* (1978) found that the detection rate for mycoplasmas in the culture method using clinical mastitis milk was significantly lower compared with that of PCR analysis. This was probably due to significant changes in the electrolyte composition of enzyme activity in the mastitis milk of affected cows, which caused rapid diminution of *Mycoplasma* in the mastitis milk, thereby influencing the detection rate

Jasper (1996) had opined that cows with latent infection might shed low numbers of mycoplasmas (perhaps intermittently)which might not be detected by use of bulk tank analysis or by use of bacteriological culture of quarter milk samples.

Ghadersohi *et al.* (1999) observed that Low recovery rate of bacteria following culture during an acute inflammatory process.

5.7 DIRECT SCREENING OF MILK SAMPLE BY PCR

Direct screening of milk sample to detect *Leptospira* and *Mycoplasma* by PCR was found to be unsuccessful.

This could be due to the presence of factors in milk that inhibited the amplification of nucleic acid by PCR. These inhibitors interfered with cell lysis necessary for extraction of DNA and thus inhibited polymerase activity for amplification of target DNA.

The casein from milk co-precipitated with the nucleic acids and inhibited the polymerase. Even though many procedures had been attempted to extract good quality template DNA from milk, most of them were unsuccessful. Culture was always more sensitive, unless a nested PCR system was used (Rosenbusch)

Hotzel *et al.* (1993) observed that the interference of proteins, fats and calcium ions present in the milk might cause false positive and false negative result for PCR.

Haymann and Hirst (2003) applied Semi Nested PCR to detect *M bovis* from milk and mucosal sample it was found that because of presumed low ratio of template DNA to total DNA in the samples and the likelihood of inhibitors particularly in milk, all samples tested were negative.

All these earlier studies lead to the fact that an alternative method of extraction which can offer a good quality DNA with minimum inhibitors as well as higher recovery rate can overcome the inherent problems associated with the extraction of target DNA from milk. Testing of PCR negative samples by semi nested PCR might detect more number of positive samples.

Summary

6. SUMMARY

Mastitis continues to be an enigmatic and costly disease of dairy cattle. Most farmers are well acquainted with traditional causes of mastitis such as *Staphylococcus* and *Streptococcus agalactia.* The widespread adoption of standard mastitis control practices has allowed many dairy farmers to control contagious forms of mastitis. While these traditional forms of mastitis are now controllable, mastitis continues to require management attention. Some fastidious organisms like *Leptospira* and *Mycoplasma* have emerged to fill the niche created by the control of contagious organisms.

In the current study an attempt has been made to detect the leptospires and mycoplasmas from bovine mastitis milk by PCR. Isolation attempts has also made from the same samples.

The molecular methods for detection of these fastidious organisms were standardized using the reference strains of *L. pomona* and *M. bovis* respectively. The genus specific primers A and B were used to amplify the leptospiral DNA, whereas primer pair MGSO and GPO 3 was used to amplify the mycoplasmasl DNA. To speciate the mycoplasmas that had detected in the study, the primer pair PpMB 920-1 and PpMB 920-2 was used .

All haemagalactic milk samples suspected for leptospirosis, and the clinical and sub clinical mastitis milk samples suspected for mycoplasmosis were used for the detection and isolation of these organisms.

Fifty milk samples pre enriched in Fletcher's semi solid medium were screened by genus specific PCR, out of which only one sample yielded an amplified product of 331 bp corresponding to leptospiral DNA. All the samples including this PCR positive sample were culture negative. The attempts to amplify the target DNA directly from the milk sample were also unsuccessful.

To confirm the etiology of *Mycoplasma* mastitis, fifty milk samples were screened by PCR. Similar to *Leptospira* the direct screening of milk sample failed to amplify the target DNA. So the milk samples pre-enriched in BHI broth were subjected to genus specific PCR. Out of the fifty samples, only one sample was positive for *Mycoplasma*. An amplified product 270 bp was detected in the positive sample. But all the attempts for isolation of organism were unsuccessful.

Results of this study indicate that PCR is a sensitive technique, which allows rapid detection of *Leptospira* and *Mycoplasma* from milk. Success of isolation depends upon the presence of viable organism in the sample which in turn depends on the aseptic collection and transit of the sample; moreover these slowly growing organisms require special laboratory conditions, and take long time to grow which will delay the clinician to adopt quick and prompt measures to avoid further complication. By adopting the PCR we can make a rapid diagnosis within 24 h the PCR can detect viable as well non viable organism. But one difficulty encountered with the PCR from milk samples was the extraction of good quality template DNA that contained fewer inhibitors. Since the rapidity and ease of PCR is largely dependent upon the extraction procedure adopted in each bio materials, the protocol followed in this study needs some modifications.

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POLYMERASE CHAIN REACTION BASED SCREENING OF BOVINE MASTITIS MILK TO DETECT *Leptospira* AND *Mycoplasma*

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

Master of Veterinary Science

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ABSTRACT

In the present study an attempt has been made to detect the *Leptospira* and *Mycoplasma* from bovine mastitis milk by PCR. Simultaneously trials were also made to isolate these fastidious organisms from these milk samples.

Direct screening of milk sample by PCR could not successfully detect the DNA of either of these organisms. All the previous studies had revealed that inhibitory factors present in the milk might interfere with the amplification of target DNA present in the milk. To overcome this difficulty initially all the milk samples were preenriched in the broth media, subsequently the target DNA was extracted.

The genus specific primer A and B were used to detect the leptospires in milk samples. Out of fifty haemagalactic milk samples screened by PCR only one sample was positive for *Leptospira*.

Fletcher's semisolid media that could support the growth of reference strain of *Leptospira pomona* was used for the isolation study. But all the attempts to recover the organism from milk sample were unsuccessful.

For the detection of *Mycoplasma*, genus specific primers MGSO and GPO3 were used. Out of the fifty samples screened only one sample was positive for M*ycoplasma*. But the *M.bovis* specific primers PpMB 920-1 and PpMB 920-2 failed to amplify the DNA that was found positive in the genus specific PCR. Since several species of *Mycoplasma* was found to be associated with the mastitis, organisms detected in this study might not be *M.bovis*.

The media used for the isolation of *Mycoplasma* were BHI broth and BHI agar. Media were standardized with the reference strain and were found to be capable of supporting the growth of organisms. But all the attempts to isolate the *Mycoplasma* from milk samples were unsuccessful.

It was concluded that PCR is a rapid and sensitive technique to detect the fastidious organisms that are difficult to isolate in the ordinary laboratory conditions. But one difficulty encountered with the PCR from milk samples was the extraction of good quality template DNA that contained fewer inhibitors. Since the rapidity and ease of PCR is largely dependent upon the extraction procedure adopted in each bio materials, the protocol followed in this study needs some modifications.