AN ASSESSMENT OF THE IMMUNOPATHOLOGICAL RESPONSE IN BOVINE MASTITIS

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences

KERALA AGRICULTURAL UNIVERSITY

Department of Pathology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY - THRISSUR

KERALA

1996

DECLARATION

I hereby declare that the thesis entitled **"AN ASSESSMENT OF THE IMMUNOPATHOLOGICAL RESPONSE IN BOVINE MASTITIS"** 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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CERTIFICATE

Certified that the thesis, entitled **"AN ASSESSMENT OF THE IMMUNOPATHOLOGICAL RESPONSE IN BOVINE MASTITIS"** is a record of research work done independently by **Sri. P. Biju**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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ACKNOWLEDGEMENT

I express my sincere gratitude to Dr. C.R. Lalithakunjamma, Associate Professor and Chairman of the Advisory Committee for the valuable guidance.

I am grateful to Dr. K.M. Ramachandran, Professor and Head, Department of Pathology and Member of the Advisory Committee for his timely advices and suggestions during the course of this study.

I wish to express my sincere thanks to Dr. K.V. Valsala, Professor, Department of Pathology and Member of the Advisory Committee for her valuable suggestions and help rendered during the course of this work.

I am very much obliged to Dr. V. Jayaprakashan, Associate Professor, Department of Microbiology and Member of the Advisory Committee for his constant encouragement and inspiring advices.

I gratefully acknowledge the valuble guidance given to me by Dr. A. Rajan, Dean, College of Veterinary and Animal Sciences.

I wish to express my sincere thanks to Dr. Sosamma Iype, Professor i/c, University Livestock Farm, Mannuty,

Dr. P. Ramachandran, Professor, Dr. Krupesh Sharma and Dr. A.M. Vahida, Assistant Professors of the Livestock Research Station, Thiruvazhamkunnu, Dr. D. Sreekumar and Dr. K.S. Anil, Assistant Professors of the Cattle Breeding Farm Thumpoormuzhy, Dr. J. Abraham, Professor, Dr. George T. Oommen and Dr. Kuttynarayanan, Associate Professors of the Meat Technology Unit, College of Veterinary and Animal Sciences, Mannuty for the valuable help and assistance rendered to me for collecting the various materials required for this work.

I gratefully acknowledge the help rendered by members of the Department of Statistics.

I wish to express my sincere thanks to all staff members of the Department of Pathology for their valuable assistance given to me for the successful completion of this study.

The help and co-operation rendered to me by Mr. Jithesh, Miss Beena, Miss Bindu, Research Associates, Dr. Jacob Alexander, Dr. S. Silamban, Dr. Vigil Anbiah, Dr. S. Nandakumar, Dr. S. Mohan, Dr. Vyas, Dr. Ajayakumar, Dr. Sulficar, Dr. Kuruvilla Varghese, Dr. Deepa Ananth, Dr. George Varghese, Dr. P.S. Jayakumar, Dr. D. Sanjay, Dr. P. Vidyadharan, Dr. Anilkumar Nair, Dr. Malarkannan, Dr. Raja and Dr. C.N. Dinesh, M.V.Sc. scholars, are gratefully acknowledged.

I am thankful to the Kerala Agricultural University for providing the financial assistance in the form of Junior Fellowship.

I am much obliged to Mr. O.K. Ravindran, C/o Peagles, Mannuthy for the neat typing of the manuscript.

I have no words to express my gratitude to my beloved parents and brother, who have been a constant source of inspiration for me.

P. BIJU

To my beloved parents

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INTRODUCTION

Mastitis, the inflammation of the mammary glands remains a serious problem for the dairy industry and greatly influences the profitability of dairy farming. It not only decreases the quantity but also impairs the quality of milk. The financial losses occurring due to mastitis are enormous. They usually occur in the form of loss of milk, cost of treatment and labour as well as increased replacement cost. In India, mastitis of cows lead to a total financial loss of Rs.889.51 crores. Sub-clinical mastitis caused a loss of Rs.603.87 crores while clinical mastitis lead to a loss of Rs.285.64 crores (Singh *et al.*, 1994).

In sub-clinical mastitis, economic loss occurs in the form of decreased milk yield. According to Blood *et al.* (1983), sub clinical mastitis causes 10-25 per cent loss in milk yield. The average prevalence of subclinical mastitis in cows was reported to be 43.90 per cent (Singh *et al.* 1994). There is paramount need to curb these losses.

During the past few decades enormous amount of work has been done and results published on different aspects of mastitis in India and other countries. A thorough knowledge of the pathological processes in the mammary gland in mastitis

is essential. In Kerala no authentic study has been made on various aspects of the disease of the mammary gland.

It is now well established that the severity of pathological changes due to mastitis is determined not only by the nature of the infecting agent, but also by the natural resistance mechanisms of the animal. Despite the tremendous work done on different aspects of mastitis, the control of mastitis is still an enigma. Studies on the etiopathology of mastitis demonstrated that the aetiological agents are present every where and it is the deficiency of immunological competency that leads to the occurrence of mastitis. Once the disease is contracted the outcome largely depend on the immunological competency of the host, which in turn depends on large number of complex variables. The dichotomy of the immune system is well-known and these systems although have different developmental pathways, co-operate to a great extent in laying out effective immune barrier in the host against invading agents. As long as the two immune systems are functioning effectively, the animal resist the infection contracted from the microenvironment and its survivability is ensured. Thus the destruction of invaders and the repair of the tissue as well as their protections require the immuno competent cells. Hence, recently scientists are taking keen interest to understand the immunopathological reactions taking place during a disease process within the host system.

Therapy can be undertaken and production loss can be reduced if the prevalence of sub-clinical mastitis is identified. If the immunopathological response in mastitis is clarified, the scope for immunomodulation as an adjunct to therapy of mastitis can be explored and mastitis treatment can be made more effective. Hence a detailed investigation was undertaken.

The present investigation was designed to study the clinico pathological features of bovine mastitis with special reference to immunopathological response.

Review of Literature

REVIEW OF LITERATURE

2.1 Prevalence

2.1.1 Kerala State

According to the disease surveillance report of the Animal Husbandry Department of Kerala (Till May, 1996) the prevalence of mastitis is on an average 5220 per month with 574 cases of sub-acute 4124 cases of acute and 522 cases of chronic mastitis.

2.1.2 India

Mastitis is one of the most important diseases among the various pathological conditions of the udder, which produces a high economic loss to the dairy farmer. In India, mastitis was first reported by Joshi in 1926 (Krishnaswamy *et al.*, 1965). Dhanda and Sethi (1962) found that the prevalence of clinical mastitis in cows was 3.9 per cent while 48.8 per cent had latent infections. Kalra and Dhanda (1964) observed that the average prevalence of mastitis in Punjab, both in rural and urban dairy establishments was 9.62 per cent in cows, in which the hind quarters of the cows were more involved.

There was increased prevalence of mastitis with increasing lactation and more prevalence in the first month of lactation (Krishnaswamy *et al.*, 1965).

Occult infection was reported in 52.14 per cent of cows. The prevalence of subclinical mastitis in cattle from three dairy farms in Rajasthan was 11.7 per cent, 21.5 per cent and 24 per cent, respectively (Bhatnagar and Mehrotra, 1969).

Rao and Naidu (1969) reported that the left half and hind quarters were affected with mastitis at greater frequency than right half and fore quarters. They could observe that the highest prevalence of mastitis was seen during the fourth lactation and fourth to sixth month after calving. Kapur and Singh (1978) found that 37 per cent of 63 cows examined had clinical mastitis. Rear quarters were infected most frequently and the occurrence of clinical mastitis was highest in the third and fourth month of lactation.

Dutta *et al.* (1988) studied the lactational prevalence rate of mastitis in exotic and crossbred cows in Thripura and found that it ranged from 16.1 to 52.2 per cent for Jersey cows and from 16.5 to 33.3 per cent for crossbred cows. They also found that the risk ratios for the development of mastitis were 1.21 to 1.98 times greater in Jersey cows than in crossbred cows.

Chandra *et al.* (1989) using California mastitis test examined 372 quarters of 94 crossbred cows and found that 29 to 84 per cent of samples were positive.

Prevalence of bovine sub-clinical mastitis was studied by Saxena *et al.* (1993). They reported that 64 per cent of the cows had sub-clinical mastitis. The highest prevalence was in the second lactation.

2.1.3 Abroad

Schalm and Ziv-Silberman (1968) had studied the incidence of mastitis.

Distribution of udder infections between cows and between quarters within cows had been studied by Smith and Coetzee (1978).

Wilson and Richard (1980) conducted a survey of mastitis in the British dairy herd and found that among 500 herds the national prevalence of subclinical mastitis as defined by the International Dairy Federation was 9.6 per cent of all quarters.

Fatality as a result of acute mastitis in cows was reported by Dejong (1987).

Incidence and types of clinical mastitis in dairy herds had been studied by Erskine *et al.* (1988).

Preez (1988) screened 365 Friesian cows in a herd and found that 15.5 per cent of quarters had subclinical mastitis,

8.3 per cent had aseptic mastitis and 39.3 per cent had teat canal infections.

Firat (1993) had studied the susceptibility of clinical mastitis in successive lactations.

2.2 Etiology

The bacterial etiology of bovine mastitis was investigated by many scientists and a number of bacterial agents have been identified.

The principal organisms associated with the mastitis were *Streptococcus agalactiae*, *Streptococcus uberis* and *Micrococcus pyogenes* (Nanjiah, 1956).

Mastitis due to *Corynebacterium pyogenes* in cows was found to be very common (Parganoker, 1956). Tannur and Malik (1968) could isolate *Corynebacterium pyogenes* from freshly drawn bovine milk.

In a study pertaining to the etiology of mastitis in cows in India, it was found that 41.2 per cent of the animals were affected with mastitis. The important etiological agents were *Staphylococcus* 11 per cent, *Streptococcus agalactiae* 14.5 per cent, *Streptococcus dysagalactiae* 7 per cent, *Streptococcus uberis* 0.5 per cent, *Streptococcus pyogenses* 0.2 per cent,

Streptococcus zooepidermicus and 1per cent *Streptococcus equisimilis* (Dhanda and Sethi, 1962).

Kalra and Dhanda (1965) reported that in both clinical and latent infections 50 per cent was due to Staphylococcus. Krishnaswamy *et al.* (1965) isolated the organisms from 91 quarter samples of milk obtained from 35 cows, and found 31.4 per cent Streptococci, 51.4 per cent Staphylococci and in 5.7 per cent cases there was mixed infection with Streptococci and Staphylococci.

Various etiological agents in three dairy farms in Rajasthan were found to be Staphylococci (50.9%), Streptococci (13.2%) and Pseudomonas (11.3%) (Bhatnagar and Meharotra, 1969). Mammary pathogens were classified as *Staphylococcus aureus* (42%), *Streptococcus agalactiae* (15%), *Streptococcus uberis* (10%) and other streptococcus (11%) (Lee and Frost, 1970). In an abattoir survey of udder pathogens from culled dairy cows, no udder pathogen was found in 44 per cent of the udders (Ziv and Nachman, 1972). Streptococcus other than agalactiae were isolated from 44.5 per cent cases, *Staphylococcus aureus* from 20.6 per cent, *Pseudomonas aeruginosa* from 8.1 per cent and *Streptococcus agalactiae* from 3.2 per cent of the udders.

Chander and Baxi (1975) examined milk samples from 304 quarters of apparently healthy cows and found that 54.4

per cent of the quarters were infected. The organisms included 68.6 per cent Staphylococcus and 16.2 per cent Streptococcus.

Kapur and Singh (1978) observed Staphylococcus as well as Streptococci as the major cause of clinical mastitis in cows.

Oliver (1987a) studied the intramammary infections in heifers at parturition and during early lactation in a herd with a high prevalence of environmental mastitis. The organisms isolated were 11.7 per cent non haemolytic coagulase negative Streptococci, 4.3 per cent Streptococci other than *Streptococcus agalactiae*, 3.2 per cent coliforms, 0.8 per cent Corynebacteria, 0.6 per cent coagulase positive Staphylococci and 0.4 per cent other mastitic pathogens.

Hamana (1988) reported the occurrence of mastitis in heifers and pointed out that normal secretion contained a few microorganisms with *Staphylococcus epidermidis* as the most predominating organism followed by *Corynebacterium pyogenes* and *Peptostreptococcus indolicus*.

Anaerobic, facultative anaerobic and microaerophilic bacteria were isolated from the teat canal of dairy cow in which a wide range of mastitic pathogens including *Staphylococcus aureus*, *Actinomyces pyogenes*, *Escherichia coli*,

Streptococcus agalactiae and 13 species of anaerobic bacteria were identified (Preez, 1988).

Isolation of environmental mastitis causing pathogens of new intramammary infection during the nonlactating period was done by Oliver (1988). Badran (1988) enumerated the factors affecting the udder susceptibility to Staphylococcal infection.

Erskine *et al.* (1988) studied the prevalence and type of clinical mastitis in dairy herds based on the somatic cell count in milk and found that the causative agents are mainly *Staphylococcus aureus* and *Streptococcus agalactiae*. In herds with high somatic cell count and coliforms in those herds with low somatic cell count. Similar observation was made by Schukken *et al.* (1989) and Berry (1994).

Various etiological agents associated with subclinical mastitis have been elucidated by Saxena *et al.* (1993) in Assam and found that out of the 74 positive milk samples *Staphylococcus aureus* was the predominating organism (40 isolates) followed by coagulase negative Staphylococci (34) Bacillus spp (21), *Streptococcus uberis* (14), Pneumococcus spp (12), Streptococcus spp (9), Corynebacterium spp (8) Micrococcus spp (6) and *Streptococcus bovis* (1).

Studies on sub-clinical mastitis in machine milked cows by Singh *et al.* (1994) showed that Staphylococci were the chief causative agents (73.08%) followed by Streptococci (14.61%) and *Escherichia coli* (5.38%). Corynebacterium, Proteus and Klebsiella spp. were also isolated.

Tuteja and Kapur (1995) studied the microbial flora producing subclinical mastitis in 179 apparently healthy cows and found 78.1 per cent to be culturally positive. Amongst 307 isolates from 697 apparently healthy quarters, Staphylococci were the most predominant (44.3%) followed by Micrococci (30%), Streptococcus (18.9%), Corynebacterium sp. (5.5%), Bacillus spp. (1%) and Enterococci (0.3%).

2.3 Cells in milk

Reports on cytology and the physiological process which govern the number and types of cells in milk are very few.

Cells in milk of healthy cows were of two types, leukocytes and epithelial cells. 'Christiansen, (1929) found true plasma cells also in milk with a high total count and he divided the cells into lymphocytes large mononuclear cells, transitory forms, eosinophils, basophils, neutrophils, mononuclear cells containing fat and epithelial cells. Differentiation of agranulocytes and granulocytes in milk was

developed by Blackburn and Macadem (1954) with a special staining technique.

The main cell seen in acute mastitic milk is the neutrophil. Its function is to phagocytose the organism and other foreign particles which cause damage to the tissues. Animals with low cell counts were easily infected with small doses of bacteria (Schalam and Lasmanis, 1963). Most of these cells found in milk had originally been stored or retained in various body sites. This could be demonstrated using labelled neutrophils (Kaneko *et al.*, 1964).

Syrstad and Ron (1979) had reported variation in somatic cell counts of milk samples from individual cows.

Factors affecting cell counts of herd bulked milks and of individual quarter milks of Rhodesian dairy cows were studied by Titterton and Oliver (1979).

Analysis of the somatic cell volume was found to aid the diagnosis of mastitis (Hoare *et al.* 1980).

Jafri (1981) had studied the milk leukocytes in sub-clinical mastitis.

Differential cell count in milk samples was been evaluated by Lalithakunjamma (1976). The study revealed that neutrophils are the major inflammatory cells in milk.

The lymphocytes in milk may be contributing gammaglobulins, which may be immunologically specific under certain conditions. Jensen and Eberhart (1981) found that the highly vacuolated mononuclear cells in milk from normal bovine mammary glands, usually designated as epithelial cells were able to phagocytose viable *Streptococcus agalactiae* and *Staphylococcus aureus* and confirmed the properties of phagocytes both *in vitro* and *in vivo*.

Hassan *et al.* (1984) had studied the susceptibility of cows and buffaloes to mastitis infection by evaluating the cells in milk.

The relationship of somatic cell in bovine milk to production and clinical episodes of mastitis was studied by Dohooi *et al.* (1984), Salsberg *et al.* (1984).

Erskine *et al.* (1988) had studied the incidence and types of clinical mastitis in dairy herds with high and low somatic cell counts.

Nader Filho and Pinto (1988) studied the variation in the number of polymorphonuclear leukocytes in milk samples from cows recently affected with mastitis.

Interpretation and practical application of cell counts in mastitis had been studied by Funke (1989).

Schukken *et al.* (1989) had reported the incidence of clinical mastitis on farms with low somatic cell count in milk.

Deluyker *et al.* (1993) had evaluated the inter relationship of somatic cell count and milk yield in a low somatic cell count herd.

Studies on the physiology of mastitis and factors affecting somatic cell count had been made by Harmon (1994).

2.3.1 Methods of cell count

Prescott and Breed (1910) introduced a method of spreading milk on a slide and counting the cells, which gained general modifications ever since. Under pathological conditions when the cell count was high the percentage of polymorphs was also high and went upto a maximum of about 90 per cent (Waite and Blackburn, 1963). Normal milk has a leukocyte formula, similar to that of blood. In mastitis milk majority of the cells was thought to be due to chemotactic stimuli which resulted from the presence of microorganisms in the udder (Cullen, 1966).

2.3.2 Significance of different levels of cell count in milk

The cell count alone cannot be taken as criterion for diagnosis of mastitis. A cell count varying from 300,000 to

10,00,000 per millilitre was found to be normal depending up on the age of the animal and stage of lactation (Little, 1938).

The differential count along with the value of total count can give very useful informations (Blackburn *et al.* 1955). Milk from normal quarters rarely contains more than 500,000 leukocyte per millilitre, and the milk from infected quarters usually exceeds this number (Plastridge, 1958).

Dattatraya (1971) studied the cells in milk of cows and buffaloes in different stages of lactation to get an idea about the milk from healthy as well as infected quarters of udder. He correlated the cell count with differential count and bacteriological examination. A count of 100,000 to 500,000 cells per ml raised suspicions and a count above 500,000 cells per ml was suggested as a conclusive evidence of mastitis. According to Geer and Pearson (1973), the cell count must be combined with cultural examination and confirmed as mastitic if any of the pathogen is present regardless of the cell count and if there were 1000,000 cells per ml with or without organisms. They found that there was a good correlation between the Streptococcal isolation and the high cell count.

Of the various indirect tests, the leukocyte count showed the highest percentage of agreement with the bacteriological examination (Chander and Baxi, 1975).

Counting of the cells in milk for diagnosing subclinical mastitis carried out on 656 samples of milk from 164 cows in Perugua by Fruganti and Valente (1980), proved that the cell count together with determination of neutrophil percentage gave more reliable diagnosis of mammary inflammation. The cell count was considered particularly valuable to detect aseptic secretory changes due to mechanical injuries which might form an early stage of mastitis.

Verhoeff and Smith (1981) evaluated the bovine serum albumin and cell counts in the diagnosis of sub-clinical udder infection.

Combination of bromphenol blue indicator test and cell count was proved to be useful for detecting infected quarters (Marschke and Kitchen, 1985).

Emmanuelson *et al.* (1987) compared some of the screening tests for detecting mastitis and found that combination of two diagnostic tests increased the predictability in most cases, although the increase was only minor.

Pednekar *et al.* (1992) evaluated some indirect tests for detecting subclinical mastitis and found that lactose estimation plus somatic cell count were the most reliable tests.

2.4 Antibiogram of the mastitic pathogens

Antibiogram of the mastitic pathogens was studied by various workers.

Gupta *et al.* (1979) evaluated the *in vitro* sensitivity of *Staphylococcus aureus* strains isolated from mastitic milk and found that nitrofurantin inhibited 99.36 per cent of *S. aureus* strains isolated from mastitic milk. Chloramphenecol, streptomycin and tetracycline inhibited the growth of 97.35, 96.81 and 94.9 per cent of *S. aureus* strains respectively. Penicillin was found to inhibit the growth of 72.61 per cent of *S. aureus*. Sulphathiazole was found to be least effective.

Antibiogram of *Staphylococcus aureus* isolates of bovine mammary origin was reported by Kapur *et al.* (1979) who found that 98 per cent of the organism were sensitive to gentamycin, spiramycin, erythromycin, cephaloridine, cloxacillin, furadantoin, chloramphenicol, neomycin, bacitracin and kanamycin. Ninety eight per cent were sensitive to streptomycin, cibramycin and ampicillin. Sensitivity to penicillin, tetracycline, oxytetracyclin and chlortetracycline

ranged from 78 to 85 per cent. 99 per cent were resistant to sulphadimidine.

Hussain *et al.* (1984) studied the sub-clinical mastitis in cows and buffaloes and identified the organisms. They also studied the drug susceptibility of the organisms.

In Kerala, Sudharma *et al.* (1985) examined the antibiogram of various agents causing mastitis and found that Staphylococci were highly sensitive to gentamycin (84%) followed by chloramphenecol (78.46%), neomycin (62.5%), tetracyclin (60.96%), furadantin (55.56%), ampicillin (50%) and penicillin (43.1%). Coliforms were sensitive to neomycin (92.6%) followed by gentamycin (85%), septran (83.33%), chloramphenecol (80.7%), tetracycline (63.79%) and furadantin (58.54%). More than 50 per cent of the isolates were resistant to streptomycin and ampicillin. Of the Streptococcal isolates 90.32 per cent were sensitive to ampicillin 82.35 per cent to chloramphenecol, 78.13 per cent to penicillin, 62.5 per cent to gentamycin and 53.85 per cent to neomycin. Only less than 50 per cent of the strains were sensitive to furadantin, tetracycline and septran.

Scharen *et al.* (1987) noticed that 279 strains of Streptococcus spp. 249 *Staphylococcus aureus* and 224 of Enterobacteriacea were resistant to 13 antibiotics. Results in 1980 were compared with those in 1986 and there were slight

difference between the two years, with a noticeable increase in the percentages of Streptococcal strains resistant to the macrolide antibiotics. These studies showed that regular evaluation of resistance pattern is necessary for proper selection of antibiotics.

Beta lactamines and rifamycin gave excellent result when sensitivity of *Streptococcus uberis* to different antibiotics was examined (Gamere *et al.* 1988).

Among 319 strains of *S. aureus* collected, Vecht *et al.* (1989) found that 38 per cent were resistant to benzyl penicillin, 21 per cent to oxytetracyclin and 11.5 per cent to streptomycin.

Char *et al.* (1993) reported that out of 595 mastitis samples tested from clinical cases of mastitis in cows, 90.08 per cent and 85.78 per cent were sensitive to gentamycin and chloramphenecol respectively.

Antibiogram of California mastitis test positive milk samples from 18 cows revealed that the isolates were highly sensitive to gentamycin, kanamycin, neomycin, oxytetracyclin and chloramphenicol but resistant to ampicillin (Jha *et al.* 1994).

Bhattacharya and Rahman (1995) studied the antibiogram of pathogens isolated from cases of bovine mastitis.

2.5 Haematological evaluation

Schalm *et al.* (1971) evaluated the packed cell volume (PCV) total leukocyte count and differential leukocyte count of blood from cows suffering from coliform mastitis. They noticed an increase in the PCV and leukopaenia in the acute state. Differential leucocyte count revealed reduced number of mature neutrophils and increased number of immature neutrophils.

2.6 Examination of udder tissues and mammary lymphnodes

2.6.1 Gross changes

Gross changes of mammary gland having mastitis varies with the type of etiological agent. Evidence of fibrosis was noticed in two of the either mammary quarters examined by Schalm and Mead (1943).

Streptococci have been found producing fibrin plugs in small ducts leading to rapid involution of the gland. The toxin of *Staphylococcus aureus* produces vasoconstriction which may result in ischemia and gangrene. Acute fulminating staphylococcal mastitis may lead to formation of purulent

fistulous tracts which discharge through the skin. The exudate produced by *Corynebacterium pyogenes* has an offensive odour. Extensive necrosis and abscess formation of the udder parenchyma and formation of fistulous tracts are common in such infections (Schalm, 1977).

Experimental induction of *Escherichia coli* mastitis in newly calved dairy cows with 500 organisms intramammarily revealed that after 10th hour there was no swelling, induration of the gland or any clots in the milk. After 15th hour of infection the udder was very hard with clots in the milk (Hill *et al.* 1979).

Escherichia coli and Streptococci produced distinct hyperaemia in the acute inflammation of mammary gland which could be revealed in cut section of the gland. In the case of acute necrotising mastitis dark blue discolouration of the skin of swollen udder could be seen. This was surrounded by a zone of slight hyperaemia. When *Escherichia coli* produced sub acute necrotising mastitis, necrosis of mammary tissue and greyish granulation tissue could be seen. In chronic purulent galactophoritis and mastitis the cisterns and ducts were filled with purulent exudate. Fibrosis was present around the ducts and in the glandular part (Hill, 1981).

Moderate swelling of the mammary gland was noticed when an inoculum containing 10^2-10^{10} number of *Staphylococcus aureus*

was inoculated intramammarily into 8 glands of 3 lactating cows (Gudding *et al*. 1983).

Jones and Hunt (1983) reported that the cut surface of the normal mammary tissue in active lactation was pale pink in colour. The sharply angled and more or less rectilinear tubules were moderately conspicuous and fit together like irregularly formed bricks in a wall and there was only very little blood supply. Cut surface of actively inflammed mammary tissue was dark pink or light red in colour. Lobules were larger in size, but less distinct and cut surface was distinctly moist and few drops of blood oozed from the cut surface. In chronic cases diffusely arranged fibrous tissue was seen which imparted a firmness to the udder.

Jones (1990) reported oedema and hyperaemia of the bovine udder having coliform infection.

Clinical reaction of streptococcal mastitis varied depending upon the extent of damage to the mammary gland. Acute exudative reaction produced fibrosis and involution. Firm painful swelling resembling fibrosis had been noticed due to stagnation of secretion. There was nodular or diffuse induration of the gland that appear firm and liver like in consistency and could be easily cut. As the inflammation proceeded there was involution of the gland, which became atrophic, dry and poorly elastic. Polypoid proliferations of

the duct epithelium caused obstruction of the milk ducts and produced accumulation of secretion resembling abscess cavities. In the early stage of the disease the glandular tissue was swollen and turgid. It could be cut easily with a knife and the swollen lobules protruded on the cut surface. The affected lobular tissue was greyish in colour and it could be easily recognised from the normal lactating tissue which was milky white in colour. Most obvious changes occurred in the cistern and large ducts only in the later stages of the disease, during which fibrosis around the duct became prominent (Hill, 1981; Mc Donald and Anderson, 1983; Jubb *et al.*, 1993).

Staphylococcal mastitis was peracute and fulminating or mild and more chronic. Affected quarters became swollen, tense and firm in acute cases, and blood stained fluid came out on cutting. When there was gangrene the tissue became blue and eventually black, softer, insensitive, cold and crepitation also developed beneath the skin of the gland (Gudding *et al.*, 1983; Mc Donald and Anderson, 1983; Jubb *et al.*, 1993).

In acute mild forms there was development of granulomatous foci which were numerous and a large proportion of gland was involved with involuted glands between them (Jubb *et al.* 1993).

In coliform infections the inflammation was often limited to only one quarter with much oedema of the gland. In some cases extensive nerosis of the tissue occurred (Jubb *et al.* 1993).

2.6.2 Histological evaluation of udder

Plastridge (1958) reported the histological changes in the udder of mastitic cows which included interstitial oedema, vacuolation and desquamation of acinar epithelium and accumalation of fibroblasts and macrophages. Such areas later became chronic and new areas of inflammation developed. Brown and Scherer (1958) reported that the histological picture of staphylococcal mastitis with nodules consisting of purulent necrotic mass with bands of connective tissue was similar to actinomycotic process. Yamagiwa *et al.* (1963) studied and classified the histopathology of mastitis in slaughtered cows as tubular, diffuse and alveolar. The mild chronic mastitis was characterised by slowly progressing pathologic changes and effect was noted in the tissue initially with normal lobules intermingled with affected lobules (Stabenfeldt and Spencer, 1965).

Lee and Frost (1970) found that the organisms were present in all parts of the glandular tissue with the exception of *Staphylococcus aureus* infection, wherein the isolates were few in the dorsal part of the gland. The

histological picture revealed scattered foci of mild changes in the alveoli and milk ducts. The inter alveolar areas were oedematous and infiltrated with neutrophils and lymphocytes.

Lalithakunjamma (1976) studied the various pathological conditions in the mammary glands of cattle and goats and reported that mastitis was the most important lesion. Catarrhal mastitis and galactophoritis of varying grades and types were the most important lesions. The cisterns and lactiferous ducts in many cases manifested productive inflammatory lesions. Interstitial mastitis characterised by fibroblastic proliferation and lymphoid reaction was also equally common. Suppurative mastitis, acute diffuse mastitis, necrotising mastitis and gangrenous mastitis were also seen in few cases.

Histology of the cow's udder at various stages of lactation was studied by Michel (1981). He found that at midlactation the acini expanded and there was little interstitial tissue. In advanced lactation, the acini were smaller and there was less enzyme activity, but an increase in interstitial connective tissue. Some lobules were involuted, others still fully active. The dry udder after first lactation was characterised by reduction in size of the acini and milk ducts, increase in the quantity of cells in the interstitial tissue and reappearance of adipose tissue. After

subsequent lactation, the dry udder exhibits proliferation of connective tissue.

Histological examination of the udder from cows having subclinical mastitis was carried out by Seffner and Haddard (1981). Inflammatory changes in the duct system were not seen in subclinical mastitis and were only seen in some cases of clinical mastitis. Galactophoritis occurred only with advanced parenchymal changes and did not lead to increased leukocyte counts in milk. High leukocyte counts during subclinical mastitis were due to exudation of leukocytes into alveoli.

A systematic histological examination of bovine mammary glands with mastitis caused by *Streptococcus agalactiae* with reference to localisation of lesions was carried out by Beherens (1984). The udder tissue from 10 cows with persistent subclinical mastitis were examined and the lesions consisted of sub acute or chronic catarrhal mastitis affecting certain parts of the udder.

Non-reactive necrosis and necrosis associated with vascular lesions affecting the whole mammary gland were noted in experimentally induced *Staphylococcus aureus* mastitis in rats (Haraldson and Jonsson, 1984).

Nickerson and Pankey (1984) observed neutrophil migration when the teat end tissues were cultured *in vitro*. The data obtained provided morphologic evidence for the following sequence of events as neutrophils passed from blood into milk. In capillaries of the sub-epithelial stroma, neutrophils adhered to luminal walls, penetrated endothelia and basal lamina, then migrated across the peri endothelial cell layer into extra vascular connective tissue adjacent to epithelial linings. The leukocytes then penetrated epithelial basal laminae and migrated between basal epithelial cells to gain access to the luminal cell layer.

Trinidad *et al.* (1990) reported the histopathology of Staphylococcal mastitis in unbred dairy heifers. Histological findings in bovine mastitis and their implications for therapy were reported by Usha *et al.* (1991).

Histopathological examination of udder tissue infected with *Streptococcus agalactiae* producing either clinically normal or abnormal milk showed that acute intrammamary foci occurred in a few acini in one or more lobules (Jubb *et al.* 1993).

2.6.2.1 Changes in supramammary lymphnodes

A number of reports about the reaction in the lymphnodes of experimental animals subjected to specific antigenic

stimulation are available recently. This helps in understanding the pathology of lymphnodes in immunological reactions.

Leduc *et al.* (1955) studied the histological changes in the regional and other lymphnodes after stimulation with conventional antigens. They used the popliteal lymphnodes for this purpose. Lymphnodes appeared larger in size than normal and contained many lymphoid follicles with active germinal centres.

Turk and Heather (1965) made a detailed histological study of lymphnodes during the development of delayed hypersensitivity following administration of soluble antigens and pointed out that the immune response was associated with stimulation of both humoral and specific cellular reactions. Outteridge (1965) mentioned the immune response of the mammary gland and regional lymphnode following antigenic stimulation.

Parrott *et al.* (1966) in an investigation on laboratory animals have shown that deep cortex and para cortical area of the lymphnodes were populated mainly by T-lymphocytes. They demonstrated proliferation of large lymphoid cells in the immune response against thymus dependent antigens. Germinal centres were shown to be thymus dependent regions associated with the production of plasma cells and humoural immune responses.

2.7 Immunopathological response

There are only very little reports about the immuno pathological aspects of mastitis. This is one of the aspect by which we can expect to have some control measures on mastitis.

Outteridge *et al.* (1965) studied the immune response of the mammary gland and regional lymphnode following antigenic stimulation.

Lascelles *et al.* (1966) demonstrated local production of antibody in the lactating mammary gland following antigenic stimulation.

Immuno histochemical study of the cellular sites of antibody formation in bovine Staphylococcal mastitis revealed that the greatest number of antibody plasmocytes were in the supramammary or regional lymph nodes. As the severity of the mastitis increased, antibody plasmocytes occurred with increasing frequency in the remotely situated lymphnodes and the spleen. Occasionally a few antibody plasmocytes with weak cytoplasmic fluorescence were found in the inter alveolar stroma of the infected mammary gland (Willoughby, 1966). Similar investigation was carried out by Smith and Porter (1967) using a semi-purified preparation of flagella of salmonella bacteria, given intramammarily.

Norcross and Stark (1970) demonstrated that successful stimulation of local antibody production is possible by intramammary injections of antigenic substances. The parenchyma of the mammary gland was extremely sensitive to infusion with any foreign substance and severe inflammation was a frequent sequele to infusion. Even then, only after a number of such infusions was a significant immune response actually attained. They emphasised that the control of mastitis by immunological means was influenced by ecology of the causative microbe.

Assessment of immunoglobulin status was made by Barber (1976).

The nature of the local immune system of the bovine mammary gland was elucidated by Newby and Bourne (1977). They examined the origin of immunoglobulins of bovine colostrum and milk using radiolabelled protein. They found that the local immune system was very inactive but could be stimulated to activity by local immunization.

Serum immunoglobulin levels in neonatal lambs were evaluated by Logan and Irwin (1977).

In the case of acute mastitis, the inflammatory response lead to the influx of actively phagocytic cells, especially neutrophils along with the exudation of serum proteins. Since

the local immune response in the udder was relatively ineffective in preventing infection, attempts at vaccination against mastitis-causing organisms have been relatively unsuccessful (Tizard, 1977).

Guidry *et al.* (1980) revealed the effect of udder inflammation on milk immunoglobulins and phagocytosis. Yang *et al.* (1980) reported a depression of B lymphocyte levels in the peripheral blood of cows with mastitis. They showed that normal cows had 33.5 ± 7.1 per cent erythrocyte antibody complement rosetting lymphocytes whereas the mastitic cows had 20.1 ± 6.0 per cent similar cells. Observation made by Ishikawa and Shimizu (1983), showed that only slight variation was there in the case of T lymphocytes.

The important role of humoral and cellular defence mechanisms in the mammary gland in the protection against infection was reported by Targowski (1983).

Buller *et al.* (1983) evaluated the bovine immunoglobulins.

Ellis and Demartini (1985) evaluated immunomorphologic and morphometric changes in pulmonary lymphnodes of sheep with progressive pneumonia.

Physiological and pathological factors influencing bovine immunoglobulin G_2 concentration in milk was reported by Caffin and Poutrel (1988). They observed an increase in milk IgG_2 in infection with major pathogens.

Kaura *et al.* (1989) reported the status of T and B lymphocytes in the peripheral blood of mastitic and healthy buffaloes. They observed a reduction in the number of peripheral B-lymphocytes whereas an increase was noticed in the case of T lymphocytes.

Nickerson (1989) evaluated the immunological aspects of mammary involution. Macrophages predominated in lacteal secretions, followed by lymphocytes and neutrophils.

Nashar *et al.* (1991) studied the immune response in the preparturient udder by infusion with soluble and particulate antigen. Ovalbumin and *Streptococcus uberis* respectively were used as antigens. Systemic profile of antigen specific lymphocyte in animals chronically exposed to Staphylococcus antigens in the mammary region was studied by Dobrzanski and Yang (1992).

Blood polymorphonuclear leukocyte chemotaxis and number of such cells during experimental *Escherichia coli* bovine mastitis were observed to be increased moderately when compared to healthy cows (Kremer *et al.* 1993).

Materials and Methods

MATERIALS AND METHODS

3.1 Milk samples

Milk samples were collected from the cows belonging to the University Livestock Farm, Mannuthy, Cattle Breeding Farm, Thumboormuzhy, Livestock Research Station, Thiruvazhamkunnu and also from those brought to the Veterinary College Hospital, Mannuthy, Kokkalai, Artificial Insemination Centres of the Veterinary College at Mannuthy and Kokkalai, District Veterinary Hospital Alappuzha, Veterinary Dispensaries in Manalur, Mundur, Thanikkudam and Uppada. Five hundred and twenty milk samples were aseptically collected at random for microbiological examination and sensitivity test, 227 samples for somatic cell count, differential cell count and chloride test and 1489 samples for California Mastitis Test to detect sub-clinical cases of mastitis. All the samples were collected in sterilized vials taking proper care to prevent contamination. Those samples brought from distant places were stored in ice while transporting. No preservatives were used.

3.1.1 Diagnosis of sub-clinical mastitis

3.1.2 *California Mastitis* Test (CMT)

California mastitis testing reagent was used for the test. The reagent was prepared as per the following formula.

Sod hydroxide	-	1.5 g
Brom Thymol Blue	-	0.5 ml
Teepol	-	0.01 g
Distilled water	-	100 ml

Approximately three ml of milk collected in the receptacles of the plastic paddle was used for the test. Equal volume of the testing solution was added and mixed by slow circular movements (Chander and Baxi, 1975).

The results were noted as

Negative	(-)	No precipitate
Doubtful	(±)	Distinct precipitate with a tendency to disappear
Slightly positive	(+)	Distinct precipitate that do not disappear
Positive	(++)	Thick mixture with precipitation and gelatinisation
Strongly positive	(+++)	Distinct gel formation which tend to adhere to bottom with a central peak on mixing

3.1.3 Chloride test

This test is based on the principle that mastitic milk contains increased quantity of chlorides. Normal chloride content of the bovine milk is 0.08 to 0.15 g%. Chloride level will be increased in mastitis milk.

The reagents include

Solution-A	Silver nitrate	1.3415 g
	Distilled water	1000 ml
	(kept in amber coloured bottle)	
Solution B	Pot. Chromate	10 g
	Distilled water	1000 ml

Procedure (Chloride test)

1. To 1 ml of milk in a test tube added 5 ml of solution A.

2. Added two drops of solution B to this mixture and mixed. Positive reaction was indicated by an yellow colouration (more than 0.15 g% of chlorides).

Reddish brown colour indicated a negative reaction when the chloride content is less than 14 g%.

3.1.4 Somatic cell count of milk

Total and differential cell count of the milk samples were also studied.

3.1.4.1 Total cell count

Using a micropipette, 0.01 ml of milk was spread on a glass slide on one square centimetre area and dried (Prescott

and Breed, 1910). Broadhurst Paley stain was used for staining the smears.

Procedure

1. Immersed the dried smear in xylene for 2 minutes, drained and dried.

2. Kept in absolute ethyl alcohol for 5 minutes.

3. Immersed in Broadhurst Paley stain for 5 seconds or longer to obtain the proper intensity of staining. Rinsed the slide in tap water dried and examined under an oil immersion microscope with a total magnification of 1000.

Milk solids stained pink, cell blue and pale blue and bacteria either deep or light blue.

Counting and calibration of microscope

Hundred microscopic fields were examined and the average cell count per field was noted (Schalm *et al.* 1971).

The average cells counted multiplied by the working factor gave the number of cells per ml of milk. The working factor was calculated by dividing the microscopic factor by the number of fields counted. The microscopic factor was

obtained by using the formula πr^2 where r is the diameter of the field seen through oil immersion objective at a magnification of 1000. Microscope with a working factor of 100,000 was used for the counting. Since 0.01 ml (1/100) of milk sample was spread over an area of 1 cm^2, the possible number of such fields which can be counted in 1 cm^2 was calculated.

3.1.4.2 Differential count of cells in milk

About 10 ml of milk sample collected from the livestock farms of the university at random was centrifuged at 3000 rpm for 30 minutes. The fat layer was removed with cotton. The supernatent was removed. Mixed the sediment and prepared films on slides and dried. Treated the slides for 2 minutes in xylene to remove the fat and then placed in methanol for 2-5 min. After drying the smears were stained with Wright's stain. Drained, dried and counted the different type of cells (Schalm *et al.* 1971).

3.2 Cultural examination for micro-organisms

All the milk samples after incubation at 37°C for 3 h were streaked in blood agar plates for detecting bacterial organisms (Merchant and Packer, 1971). The blood agar plates were examined after 24 h incubation at 37°C and the colonies were identified. Those plates showing no growth were

incubated for another 24 h, or longer to detect the presence of slow growing organisms.

3.2.1 Identification of the bacterial agents

The organisms were tentatively identified upto the genus level based on the results of the following tests and the growth on selective media.

1. Grams reaction
2. Catalase test
3. Coagulase test

The procedure mentioned by Cowan (1974) was followed for the above tests.

Special media

1. Mannitol Salt Agar
2. Mac Conkey Agar
3. China Blue Lactose Agar
4. Edwards Medium
5. Streptococcus Selection Agar

3.2.2 Antibiogram

The bacterial colonies were subjected to sensitivity test as per the procedure mentioned by Cowan (1974). Nutrient

broth was used for the multiplication of the organism, which was then swabbed on Muller-Hinton agar. Antibiotic sensitivity discs supplied by Hi-Media were used for the study. Discs of the following drugs were used.

1. Penicillin (10 units)
2. Ampicillin (10 mcg)
3. Chloramphenicol (30 mcg)
4. Gentamycin (10 mcg)
5. Streptomycin (10 mcg)
6. Oxytetracyclin (30 mcg)
7. Pefloxacine (5 mcg)
8. Co-trimoxazole (25 mcg)

The results were read after 24 h.

3.3 Healthy non-mastitic cows

All the above tests were also done on 40 milk samples collected from all the four quarters of 10 apparently healthy non-mastitic cows.

3.4 Haematological Evaluation

3.4.1 Erythrocyte sedimentation rate (ESR) and packed cell volume (PCV)

ESR and PCV were determined using the Wintrobe tube, as mentioned by Wintrobe (1974).

3.4.2 Haemoglobin

Haemoglobin was estimated by acid haematin method, using Sahli's haemoglobinometer (Benjamin, 1978).

3.4.3 . Total leucocyte count

It was also estimated as per the method described by Schalm *et al.* (1975).

3.4.4 Differential leukocyte count

It was done using Wright's stain as per the method described by Benjamin (1978).

3.4.5 Determination of total protein in serum

Total serum protein was estimated by Biuret method (Inchiosa, 1964).

3.4.5.1 Preparation of the standard curve

Commercial bovine gammaglobulin (Sigma Chemicals, USA) was dissolved in pooled pre colostral calf serum to get a final concentration varying from 2 mg/ml to 100 mg/ml. They were diluted with distilled water at the ratio of one in two. The optical densities were determined in spectrophotometer. The optical density was plotted in a graph paper against the

concentration of gammaglobulin and a standard curve was prepared.

3.4.6 Determination of gammaglobulin in the serum

Zinc sulphate turbidity test (Mc Ewan *et al.* 1970) was followed with suitable modification.

A working solution of zinc sulphate (Zn SO_4 H_2O) was prepared by diluting 4.1 ml of 5% zinc sulphate to one litre of freshly boiled and cooled double distilled water, to give a final concentration of 205 milligrams per millilitre.

Tubes were arranged in three rows with the number of tubes in each row equal to that of the samples to be used. For each serum sample three tubes were kept. Six millilitres of zinc sulphate solution were poured into the first two tubes and a similar amount of distilled water in the third row, which was taken as control. Diluted serum (one in two in distilled water) was poured into corresponding tubes in the first two rows and to the corresponding tube that contains 6 ml of distilled water. Tubes were gently shaken and allowed to stand at the room temperature for one hour. The turbidity developed in each tube was then read in a spectrophotometer (Model CL-27 of Elico) at a wave length of 490 nanometer. The null adjustment was made against zinc sulphate solution. The reading of the control was subtracted

from the average readings of the test solution to arrive at the optical density of the individual sample. The optical density was then converted into gammaglobulin content (mg/ml of serum) using a standard curve.

3.4.7 Enumeration of acid alpha naphthyl acetate esterase positive cells in the peripheral blood

Number of acid alpha naphthyl acetate esterase (ANAE) positive cells in the peripheral blood was determined.

Blood smears were prepared from the peripheral blood. The smears were fixed immediately after preparation before drying. The fixative contained six parts of acetone and four parts of 0.038 M sod. nitrate (pH 5.4). Smears were immersed in the fixative for thirty seconds, rinsed in distilled water and air dried (Giorno and Beverly, 1980). Smears were labelled and stored at room temperature.

A reaction mixture was prepared for staining the smears. In 40 ml of 0.067 M phosphate buffer (pH 5.0) 2.4 ml of hexazotized pararosaniline and 10 mg of alpha naphthyl acetate (Loba) dissolved in 0.4 ml acetone were added and the final pH of the reaction mixture was adjusted to 5.8 with 2N sod. hydroxide.

The hexazotized pararosaniline was prepared by mixing equal volumes of two solutions. (1) Freshly prepared 4% sod. nitrite in distilled water and (2) one gram of pararosniline hydro chloride (Sigma chemicals) dissolved in 20 ml of distilled water and 5 ml of 12N hydro chloric acid. The hexazotized pararosaniline which formed was shaken and then allowed to stand for one minute before adding it to the reaction mixture (Knowles *et al.* 1978).

The slides were incubated in the reaction mixture for eighteen to twenty one hours at room temperature and then rinsed thoroughly with distilled water. The slides were counter stained with one per cent toludine blue for forty five to sixty minutes. The slides were then washed with distilled water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and mounted in DPX. The slides were observed under oil immersion objective of a microscope. Those lymphocytes with localised orange and nodular reaction product in the cytoplasm were considered as positive cells (T lymphocytes). The number of positive cells in every hundred cells were counted and recorded.

3.5 Udder tissues and supramammary lymphnodes

The udder tissues and supramammary lymphnodes were collected at random from the Municipal Slaughter House,

Thrissur, Meat Technology unit of the Veterinary College and from animals brought to the Department of Pathology for post mortem examination. The tissues were fixed in 10% neutral buffered formalin and paraffin sections of 5 um thickness were taken. Routine staining with Harri's haematoxylin and eosin was done for evaluation of the histological changes. Special stains using Masson's Trichrome and Taylor's Brown'Brenn modified gram stains as described by Sheehan and Hrapchack (1980) were done as and when required. A total of 336 udder tissues and 34 supramammary lymphnodes were examined for macroscopical and microscopical changes.

RESULTS

4.1 Sub-clinical mastitis

4.1.1 California mastitis test

A total of 1489 samples from 407 cows were examined for the presence of subclinical mastitis using California mastitis test (CMT). Out of these 331 (22.23%) samples were positive and 893 (59.98%) were negative. The remaining 265 samples gave a doubtful result. Depending upon the intensity of reaction 189 (57.09%) cases were '+' 97 (29.31%) were '++' and 45 (13.59%) were '+++'.

4.1.2 Somatic cell count

Two hundred and twenty seven samples were examined for somatic cell count (Fig.4,5), which comprised of the 193 samples screened for sub-clinical mastitis, and 34 clinically positive samples. The range of somatic cell and the mean cell count in each category is shown in Table 1.

4.1.3 Differential count of the cells in milk

Out of the 227 samples of milk examined, the range of epithelial cell was from 6-9 per cent in 38.46 per cent of cases and 14-40 per cent in the remaining 61.54 per cent cases.

Neutrophils ranged from 31-45 per cent in 23.07 per cent of samples and 56-83 per cent in 76.93 per cent cases.

Lymphocytes ranged from 7-18 per cent in 53.84 per cent of samples and in 46.16 per cent cases, it ranged from 25-33 per cent.

Only 61.5 per cent cases had monocytes, in which it ranged from 1-13 per cent.

4.1.4 Chloride test

The 227 samples subjected for CMT and somatic cell count were used for chloride test. One hundred and forteen samples gave positive result for chloride test (contained more than 0.15 g% of chlorides) and 79 samples contained less than 0.15 g% of chlorides indicating a negative result.

4.2 Cultural examination

A total of 520 milk samples which included 193 samples tested for sub-clinical mastitis and 34 clinically positive samples were subjected for the presence of various bacterial organisms. Out of these, 186 samples including 16 CMT negative samples, 10 doubtful samples and 48 CMT positive samples were culturally positive (35.76%) and the rest 334

(64.24%) that included 56 CMT negative samples, 9 doubtful samples and 54 CMT positive samples were negative.

Staphylococci were the predominating organism (50%) found in the cultural examination. Out of the total 93 Staphylococci isolated, 60.22 per cent were coagulase positive (Fig.6) whereas the remaining 39.78 per cent were coagulase negative.

Other organisms isolated were Streptococci (Fig.7), Coliforms, Corynebacterium, Pseudomonas, mixed organisms and gram positive bacilli in 41 (22.04%), 16 (8.6%), 12 (6.5%), 4 (2.15%), 18 (9.67%) and 2 (1.07%) cases respectively.

4.2.1 Antibiogram of the various bacterial agents

The sensitivity of the isolated bacterial agents to various antibiotics were as follows.

Coagulase positive Staphylococci were more sensitive to gentamycin (98.21%) followed by pefloxacine (96.42%), chloramphenicol (91.07%), streptomycin (82.14%), cotrimoxazole (80.35%), ampicillin (73.21%), oxytetracycline (71.43%) and penicillin (60.71%).

Coagulase negative Staphylococci were more sensitive to chloramphenicol and gentamycin (100%) followed by pefloxacine (94.59%), streptomycin and cotrimoxazole (91.89%),

oxytetracycline (75.68%) and penicillin and ampicillin (67.5%).

Majority of the Streptococci was equally sensitive to gentamycin and chloramphenicol (97.56%) followed by pefloxacin (92.68%), cotrimoxazole (87.8%), penicillin, ampicillin and streptomycin (78.05%) and oxytetracycline (63.42%).

Coliforms, the third major pathogen were sensitive to gentamycin (93.75%), chloramphenicol and pefloxacin (87.5%), cotrimoxazole (68.75%), streptomycin (62.5%) and oxytetracyclin (56.25%). All the coliforms were resistant to ampicillin and penicillin.

Corynebacterium was found to be sensitive to pefloxacin, gentamycin and chloramphenicol (100%) followed by cotrimoxazole and ampicillin (83.33%), streptomycin (75%) and penicillin (66.66%). Majority of them were resistant to oxytetracycline.

Pseudomonas were susceptible to pefloxacine and gentamycin (100%) and next in the order was oxytetracycline and chloramphenecol (75%), cotrimoxazole and streptomycin (50%). Seventy five per cent of them were resistant to both ampicillin and penicillin.

All the gram positive bacilli were sensitive to pefloxacine, streptomycin, oxytetracycline, cotrimoxazole, gentamycin, chloramphenicol and ampicillin and 50 per cent were resistant to penicillin.

The various agents causing mixed infection included Staphylococci and Streptococci, Staphylococci and Coliforms, Staphylococci and gram positive bacilli, Staphylococci and Corynebacterium, Streptococci and Coliforms, Streptococci and Pseudomonas, Corynebacterium and Coliforms, Coliforms and gram positive bacilli. The antibiogram of these are given in Table 2.

4.3 Haematological evaluation

Six animals each suffering from acute and sub-clinical mastitis, seven animals suffering from chronic mastitis and ten apparently healthy animals were subjected for haematological evaluation.

4.3.1 Packed Cell Volume (PCV) and Erythrocyte Sedimentation Rate (ESR)

The result of examination of packed cell volume (PCV) and Erythrocyte sedimentation rate (ESR) are given in Table 3.

4.3.2 Haemoglobin (Hb)

The result of examination of Haemoglobin (Hb) is given in Table 3.

4.3.3 Total leucocyte count

Leukocytopaenia was noticed in sub-clinical cases. Mean leukocyte count of the animals suffering from acute mastitis was 8630 ± 1130 cells per microlitre of blood whereas those having sub-clinical and chronic mastitis had leukocyte counts of 3990 ± 560 and 12030 ± 1631.56 respectively and the normal non mastitic cows had 10100 ± 560 leukocytes per microlitre of blood (Table 4).

4.3.4 Differential count

When the differential count was examined, neutrophils were more in the case of animals suffering from clinical mastitis (Table 4).

4.3.5 Total protein

The serum protein was almost in the normal range except in sub-clinical cases where a slight increase was noticed. Total serum protein in acute, sub-clinical and chronic cases of mastitis was 9.14 ± 1.03, 11.03 ± 0.89 and 8.47 ± 0.86 gm%

respectively and in healthy animals it was 8.5 ± 0.52 gm% (Table 5).

4.3.6 Gammaglobulin level

Serum gammaglobulin was more in the case of animals suffering from sub-clinical mastitis. In acute and chronic cases, it was more or less equal. Healthy animals had a gamma globulin level of 41.55 ± 2.98 mg/ml of serum and those in acute, sub-clinical and chronic stage of mastitis had 49 ± 5.37, 64.08 ± 5.13 and 49.07 ± 7.79 mg/ml of gammaglobulin respectively (Table 5).

4.3.7 T-lymphocyte count

T-lymphocytes were more than the normal range in acute, and chronic cases. Mean percentage of ANAE positive cells (Fig.23) in healthy animals was 31.3 ± 1.42. In acute stage of mastitis it was 38.33 ± 0.8. In sub-clinical and chronic it was 29.83 ± 1.54 and 35.00 ± 0.93 cells respectively. The values are presented in Table 6.

4.4 Examination of the mammary gland

4.4.1 Gross changes

Out of the 336 quarters examined, 18 had very clear gross lesions. All those glands were swollen and hard. They could be cut easily and the lobulations were very prominent. Cut

section of one of the quarters revealed hyperaemia. Seven quarters revealed a mottled appearance or marbling of the parenchyma when cut.

All the other glands were having almost the same colour and consistency as that of healthy glands. No odema or necrosis could be detected in any of the quarters.

4.4.2 Histological changes

Out of the 336 mammary gland tissues examined histologically, 271 were having various stages of inflammatory reactions. Out of these 271 reacting glands 207 were lactating and 64 were in different stages of involution.

Majority of the lactating glands (37.68%) were having inflammatory cells in the acini as well as interstitium whereas 33.37 per cent had inflammatory cell in the acini alone, 13.04 per cent had inflammation of interstitium and entire lobules were affected in 14.49 per cent cases. In 2.42 per cent cases inflammation of the lactiferous ducts was noticed.

In the case of non lactating glands 53.13 per cent had mononuclear cell infiltration in the interstitium. Inflammatory cells were seen in the acini as well as interstitum in 20.31 per cent of cases. Acini alone contained

inflammatory cells in 14.06 per cent cases. In 9.38 per cent of the glands there was inflammation of the entire lobules. Lactiferous ducts were inflammed in 3.13 per cent of cases.

4.4.2.1 Lactating glands

Acute and chronic changes were seen in the lactating mammary glands.

Acute changes

The acini contained inflammatory cells, especially neutrophils, which varied from a few cells to those almost filling the acini (Fig.9). Desquamation of the acinar lining cells was a common feature. The desquamated acinar lining cells and the inflammatory cells were seen either freely or embedded in the thick colloidal material present in the alveoli. Necrosis of the inflammatory cells as well as the acinar lining cells was a major change noticed (Fig.10,11). The acini which contained numerous inflammatory cells and exudate showed flattening of the lining cells. Presence of fibrin was noticed in few glands. Lymphocytes and monocytes could also be noticed in few of the acini.

In those tissues which showed only mild inflammatory changes, the acinar lining cells were almost normal. The inflammatory cells in some of the acini of such tissues showed

mild degenerative changes, which was characterised by less intensity of staining.

The inter acinar and inter lobular connective tissue were very little in the lactating gland. Few neutrophils were seen in some areas. Dialatation of the inter acinar blood vessels was seen in few tissues. In some other tissues there was damage to the blood vessel wall, which caused escape of blood into the interstitial space.

Infiltration with inflammatory cells mainly of mononuclears was noticed in the sub epithelial areas of the lactiferous ducts, producing galactophoritis. Inflammatory cells, especially neutrophils were also seen in the lumen of such ducts (Fig.12,18). Such cells appeared intact in few alveoli, whereas in others there was varying degree of degeneration and necrosis of the inflammatory cells.

Presence of corpora amylaceae were a common feature of many of the tissues. They were present in all locations but mostly within the acini. In some tissues these structures were present in almost all the acini. Corpora amylaceae were round lamellated bodies with marginal degree of calcification and staining pink with haematoxylin and eosin. The dark blue bodies were positive for calcium staining. In some bodies, only the periphery was dark blue and the centre was light blue

or pink in colour. There was no cellular reaction around these bodies.

Chronic changes

Chronic changes of inflammation in the lactating glands included changes like presence of mononuclear cells in the acini and interstitium and thickening of the walls of the lactiferous ducts.

Complete destruction of the acini and inflammatory cells in the interstitium along with fibroblasts were noticed in certain tissues (Fig.17). Such changes were intermingled with normally secreting acini in few lobules. Destruction of the entire lobule was seen in few cases (Fig.14).

Vacuolation of the desquamated acinar cells was noticed in few cases. Lymphoid aggregation was noticed in some areas of the interstitium (Fig.15).

Severe hemorrhage in the interstitium was noticed in 7 cases. The acinar lining cells were severely affected in such cases and the cells were seen detached from the basement membrane. Pyknosis of the nuclei of such lining cells was also noticed.

Intensity of inflammation of the lactiferous duct varied from gland to gland.

Polypous projections of the lining epithelium were seen into the lumen of some of the lactiferous ducts. There were collections of lymphoid cells in the subepithelial space of these ducts (Fig.16).

4.4.2.2 Involuted glands

Intense fibrous tissue proliferation was seen in the interacinar and interlobular areas and the acini were reduced in number and size by the proliferating fibrous tissue. Mononuclear cell infiltration in the interstitium was the major feature of the involuted glands (Fig.13). Few polymorphonuclear cells were also seen in some areas. Infiltration of mononuclear as well as polymorphonuclear cells was also seen in the acini of some of the glands. Desquamation of the lining cells was seen in few acini (Fig.8). Vacuolation was a feature of some of the intact and desquamated acinar cells. Thickness of the interstitial tissue varied in different glands.

Apart from the above changes, some of the involuted glands showed lesions like damaged inter lobular duct with disruption of the lining cells. Fluid exudation into the acini as well as the disruption of architecture was noticed in few glands. Few sections showed extensive fibrous tissue

proliferation. Galactophoritis which varied in intensity was a feature in a very few glands. Occlusion of the ducts as well as the acini with the lining cells was noticed in three cases. Polypous projection of the epithelium into the acinar lumen could be seen in several cases.

In addition to the above changes some of the glands which were undergoing involution were also examined. Infiltration into the acinar lumen as well as in the interstitium with polymorphonuclear and mononuclear cells was a common feature in majority of such glands. Normally secreting lobules contained mainly polymorphonuclear cells in the lumen of the alveoli. Eosinophilic secretion along with polymorphonuclear cells were seen in the non secreting lobules. The lining cells of the ducts were damaged in a few cases. Collection of mononuclear cells in the subepithelial space, obliteration of the alveoli and fibrosis of the inter alveolar space were the features noticed in a few cases.

Increased amount of fibroblasts were seen in many cases which also contained focal collection of polymorphonuclear cells. Obliteration of the acini was noticed as a common feature in these cases. Corpora amylaceae were seen in the acini of many glands. Galactophoritis of varying severity was also seen in many glands which was characterised by collection of mononuclears in the subepithelial space and

papillary projection of the epithelia into the lumen of these ducts.

4.4.3 Changes in the supramammary lymphnodes

4.4.3.1 Gross changes

Lymphnodes collected along with those glands having mastitis were enlarged in size, Oedematous and soft in consistency. It could be cut easily as compared to those from normal glands.

4.4.3.2 Histological changes

There was marked expansion of the cortex by numerous lymphoid follicles in various stages of activity (Fig.19). Active secondary follicles contained germinal centres which comprised of two zones, a basal zone which was dense and consisted of large lymphoblasts and a more loosely arranged apical zone that contained a few lymphocytes (Fig.21).

In majority of the cases the sub capsular sinuses were moderately distended and contained lymphocytes (Fig.22).

Medullary cords were moderately to markedly widened and contained mainly lymphocytes and lymphoblasts with prominent nucleoli (Fig.20). Number of plasma cells in the medullary cords varied in majority of the nodes.

Table 1. Results of milk samples subjected to various tests

Stage of mastitis	No. of sample subjected for all the tests	CMT		Somatic cell count		Chloride Test		Cultural examination	
		Result	No. of samples	Range	Mean	below 0.15 g%	above 0.15 g%	+	-
Sub clinical	193	-	72	106000-450000	217180.56 ± 8522.8067	79	114	16	56
		* DF	19	247000-477000	346894.74 ± 15681.018			10	9
		+	24	256000-1019000	541500.00 ± 51756.012			13	11
		++	42	554000-2403000	991166.67 ± 64124.723			13	29
		+++	36	984000-6306000	2410027.80 ± 221255.17			22	14
Acute	13			468000-2985000	1164769.20 ± 195875.19			11	2
Chronic	21			160000-775000	411238.1 ± 36747.329			16	5
Healthy	40	-	40	109000-400000	223675.00 ± 12149.939	40	-	2	38

* Doubtful

Table 2. Sensitivity results of mixed infection

Type of organisation	No. of org. / Total	Pf	S	O	CO	G	C	P	A
Staphylococci + streptococci	5/18	80	80	60	80	100	100	40	40
Staphylococci + coliforms	3/18	100	66.66	66.66	66.66	100	100	66.66	66.66
Staphylococci + corynebacterium	3/18	100	66.66	66.66	33.33	100	100	66.66	66.66
Staphylococci + gram positive rods	2/18	100	100	100	50	100	50	100	100
Corynebacterium + coliforms	2/18	100	100	50	100	100	100	R	R
Streptococci + coliforms	1/18	100	R	R	100	100	100	R	R
Streptococci + pseudomonas	1/18	100	R	100	100	100	100	R	R
Coliforms + gram positive rods	1/18	100	100	R	100	100	100	R	R

Pf - Pefloxacin
S - Streptomycin
O - Oxytetracyclin
Co - Cotrimoxazole
G - gentamycin
C - Chloramphenecol
P - Penicillin
A - Ampicillin
R - Resistant

Table 3. Erythrocyte sedimentation rate (ESR), Packed cell volume (PCV) and Haemoglobin concentration (Hb) in mastitic (acute, chronic and sub-clinical) and healthy non mastitic cows

Sl. No.	Stage of mastitis	ESR (mm/hr)	PCV (%)	Hb gm %
1.		0	34	10.2
2.		0	18	7.8
3.	Acute	0	32	10.2
4.		0	43	15.2
5.		0	32	11.0
6.		0	31	11.6
1.		0	22	8.0
2.		0	23	8.0
3.	Sub clinical	0	33	10.8
4.		0	28	8.4
5.		0	30	12.1
6.		0	34	11.0
1.		0	33	11.2
2.		0	25	8.2
3.	Chronic	0	30	11.4
4.		0	38	13.0
5.		0	28	8.2
6.		0	28	8.4
7.		0	30	10.8
1.		0	28	10.8
2.		0	39	12.6
3.		0	42	14.0
4.		0	30	11.2
5.	Healthy	0	46	16.2
6.		0	33	12.0
7.		0	36	14.0
8.		0	40	14.2
9.		0	44	13.0
10.		0	32	11.2

Table 4. Total and differential leukocyte counts in the blood of mastitic (acute, chronic and sub-clinical) and healthy non-mastitic cows

Sl. No.	Stage of mastitis	Total leucocyte No. of cells/mm³	Differential count						
			Myelo. %	Stab. %	Seg. %	Juv. %	Lym. %	Mono. %	Eosin %
1.		7200	35	3	4	0	20	8	0
2.		5050	11	2	63	0	17	2	0
3.	Acute	10900	11	2	63	0	17	2	5
4.		11950	3	7	58	0	30	1	1
5.		6400	10	4	20	4	56	2	4
6.		10300	2	7	44	1	43	2	1
1.		4600	14	6	27	9	37	7	0
2.		3800	21	10	34	0	32	2	1
3.	Sub	2400	70	1	8	1	15	1	4
4.	clinical	3600	15	3	14	0	64	4	0
5.		3150	6	10	62	0	19	1	2
6.		6400	5	2	52	0	31	3	7
1.		10350	1	0	58	1	37	1	2
2.		10570	10	3	56	2	24	1	4
3.	Chronic	16750	3	1	79	0	17	0	0
4.		7070	20	17	22	0	29	2	10
5.		19300	9	9	49	2	28	0	3
6.		11150	17	4	13	2	56	3	5
7.		9200	4	6	62	1	22	1	3
1.		7060	11	3	20	0	64	2	0
2.		10900	9	2	26	0	62	1	0
3.	Non	11950	10	4	48	4	30	2	2
4.	mastitic	10350	12	7	36	0	44	0	1
5.		9400	7	3	33	1	52	1	3
6.		10560	2	1	24	1	71	0	1
7.		11440	3	2	45	1	47	0	2
8.		10850	3	4	38	0	53	1	1
9.		70900	1	1	39	2	55	2	0
10.		11820	2	2	42	4	47	1	2

Myelo - Myelocyte
Stab - Stab cells
Seg - Segmented cells
Juv - Juvenile cells
Lym - Lymphocyte
Mono - Monocyte
Eosin - Eosinophil

Table 5. Serum total protein and gamma globulin in mastitic animals and healthy non mastitic cows

Sl. No.	State of infection	Total protein (mg/ml)	Gammaglobulin (mg/ml)
1.	Acute	68	34.0
2.		109	57.0
3.		116	49.5
4.		64	32.5
5.		118	65.0
6.		73.5	56.0
1.	Sub clinical	120	62.0
2.		118	70.0
3.		112	82.0
4.		110	70.5
5.		69	50.0
6.		133	50.0
1.	Chronic	66	35.5
2.		133	82.0
3.		83	24.0
4.		64	39.5
5.		84	39.5
6.		83	52.5
7.		80.3	70.5
1.	Non mastitic	76	32.5
2.		66	30.5
3.		82	50.0
4.		76	32.5
5.		102	54.5
6.		80	39.5
7.		110	53.0
8.		106	50.5
9.		90	38.0
10.		62	34.5

Table 6. Alpha Naphthyl Acetate Esterase positive lymphocytes (T cells) in the peripheral blood of mastitic (Acute, chronic and sub-clinical cases) and non-mastitic cows(healthy)

Sl. No.	Stage of mastitis	Blood smear-counted 200 cells			
		ANAE +ve	%	ANAE -ve	%
1.		74	37	126	63
2.		74	37	126	63
3.	Acute	78	39	122	61
4.		72	36	128	64
5.		80	40	120	60
6.		82	41	118	59
1.		52	26	148	74
2.		60	30	140	70
3.	Sub-clinical	62	31	138	69
4.		50	25	150	75
5.		64	32	136	68
6.		70	35	130	65
1.		64	32	136	68
2.		72	36	128	64
3.	Chronic	64	32	136	68
4.		70	35	130	65
5.		70	35	130	65
6.		78	39	122	61
7.		72	36	128	64
1.		74	37	126	63
2.		60	30	140	70
3.		72	36	128	64
4.		72	36	128	64
5.	Healthy	52	26	148	74
6.		50	25	150	75
7.		60	30	140	70
8.		68	34	132	66
9.		62	31	130	69
10.		52	26	148	74

Fig.1 CLASSIFICATION OF POSITIVE RESULT OF CALIFORNIA MASTITIS TEST

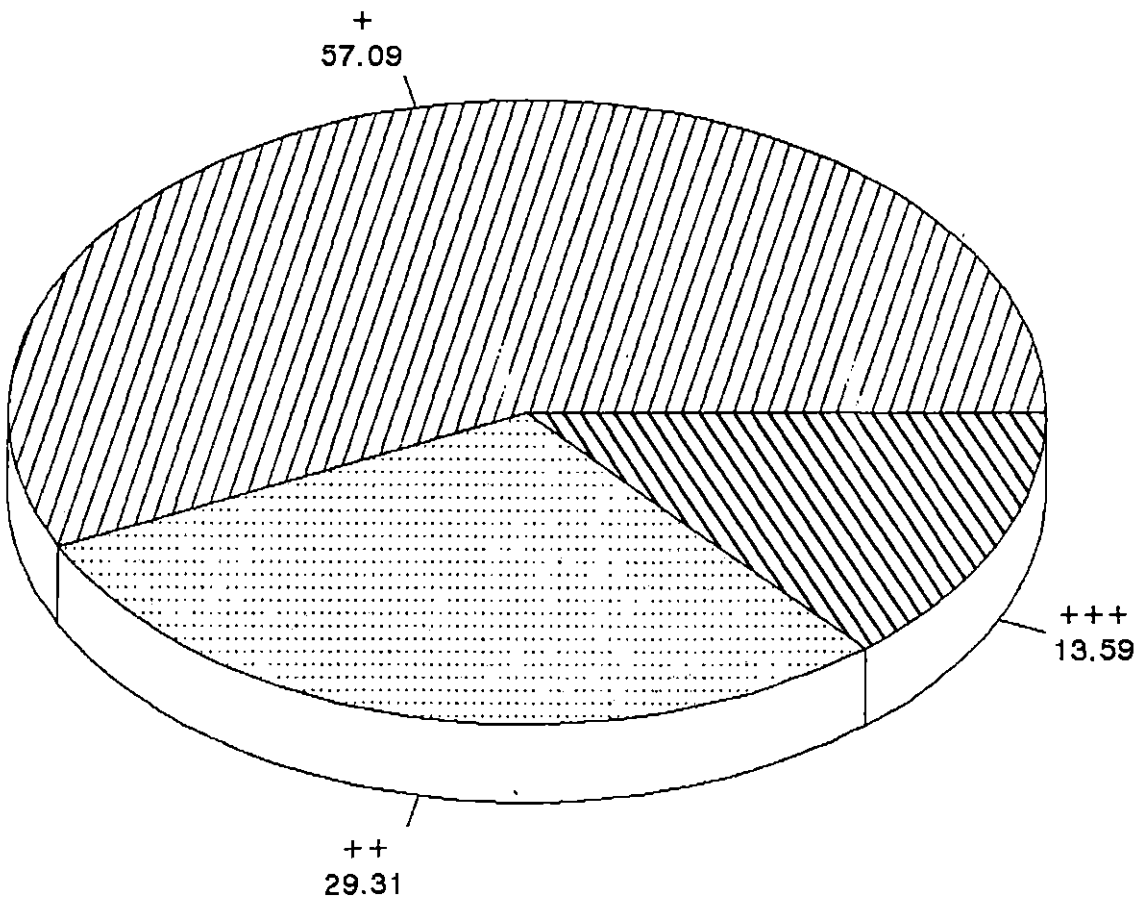
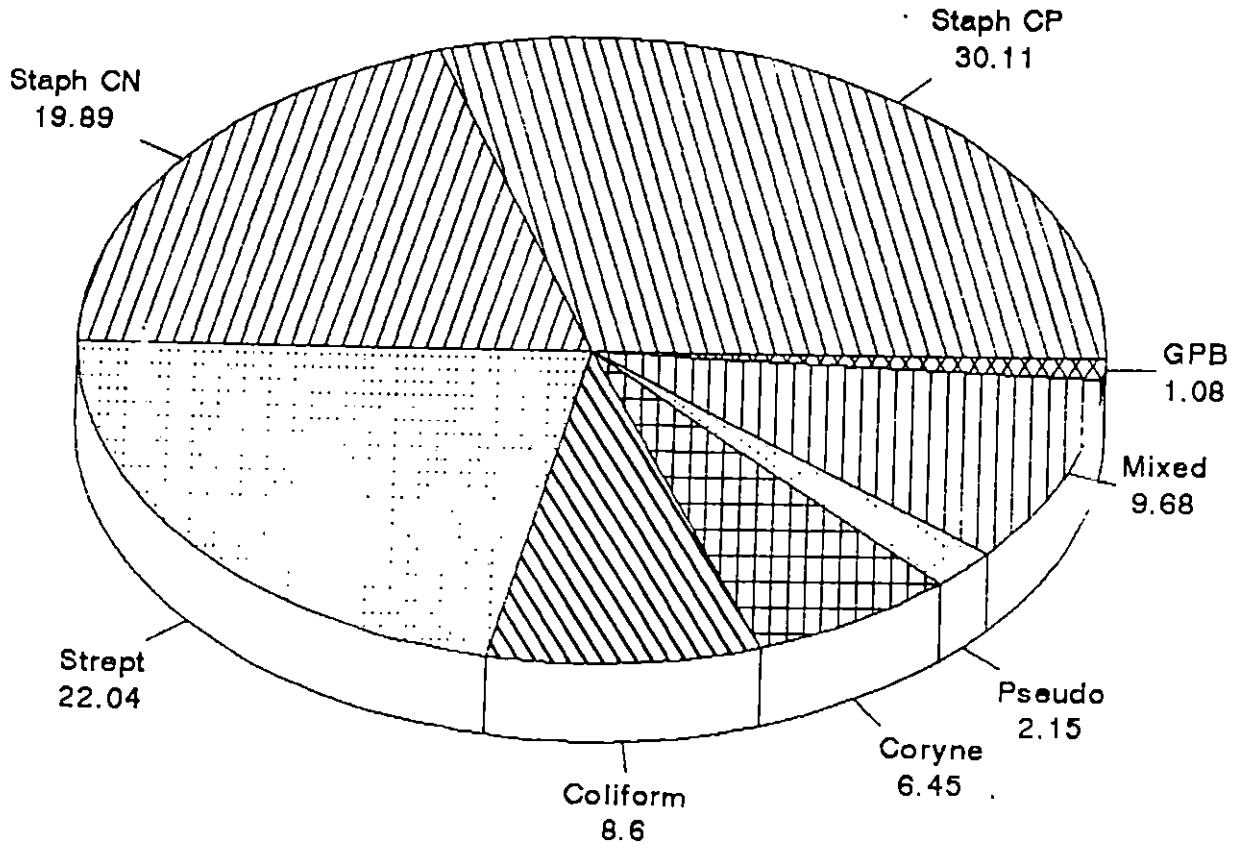


Fig.2 PERCENTAGE OF THE VARIOUS BACTERIA IN POSITIVE CULTURES



Staph CP - Staphylococci - coagulase positive

Staph CN - Staphylococci - coagulase negative

Coryne - Corynebacterium

GPB - Gram positive bacilli

Pseudo - Pseudomonas

Strept - Streptococci

Fig.3 STANDARD CURVE FOR GAMMAGLOBULIN

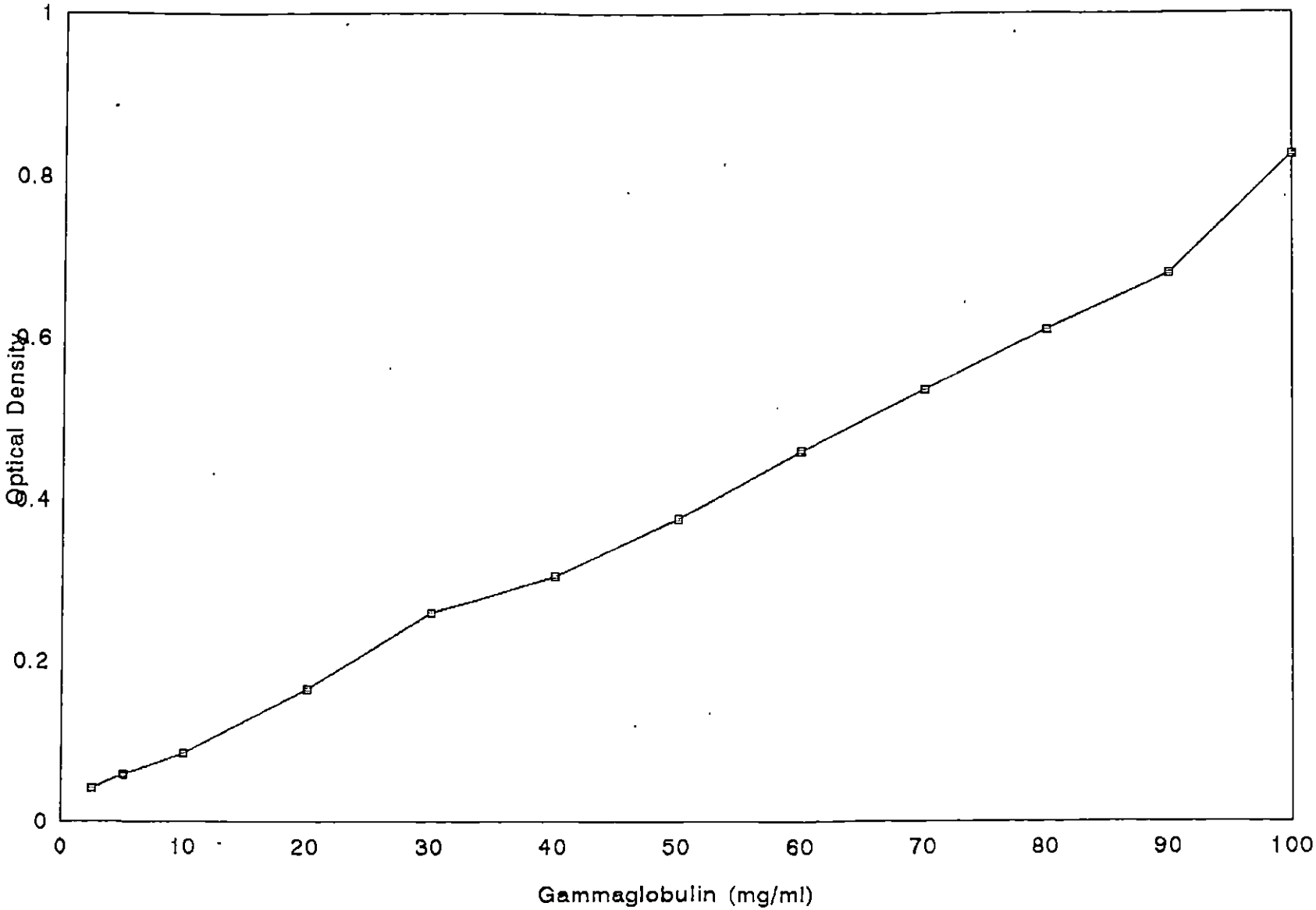
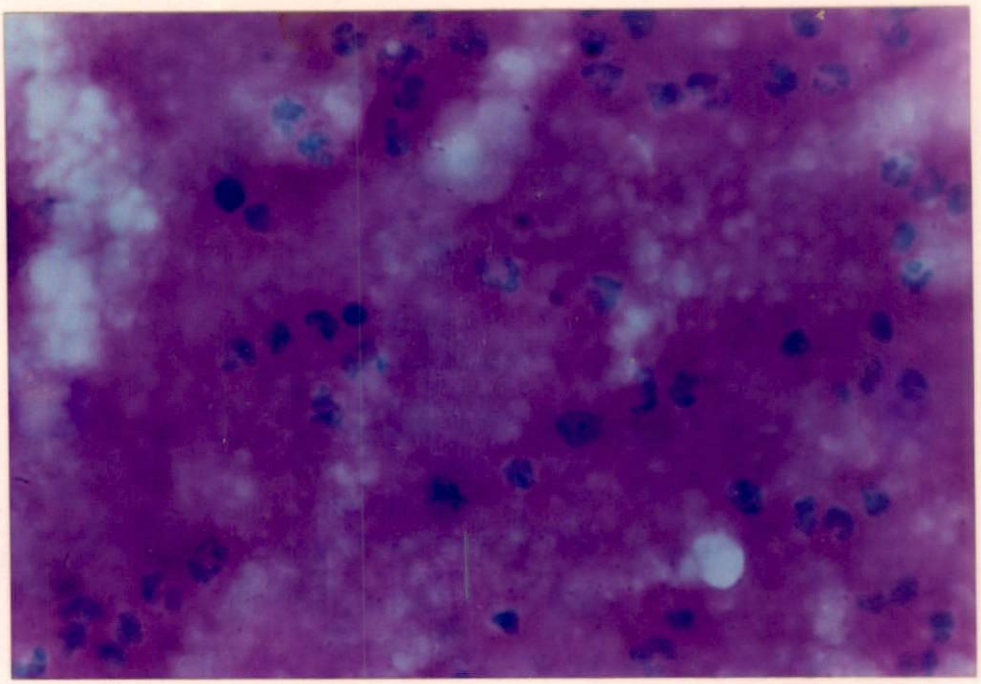


Fig.4 Smear of milk containing various somatic cells. Broadhurst - Paley stain x 1000

Fig.5 Smear of milk containing numerous polymorphonuclear cells. Broadhurst - Paley stain x 1000



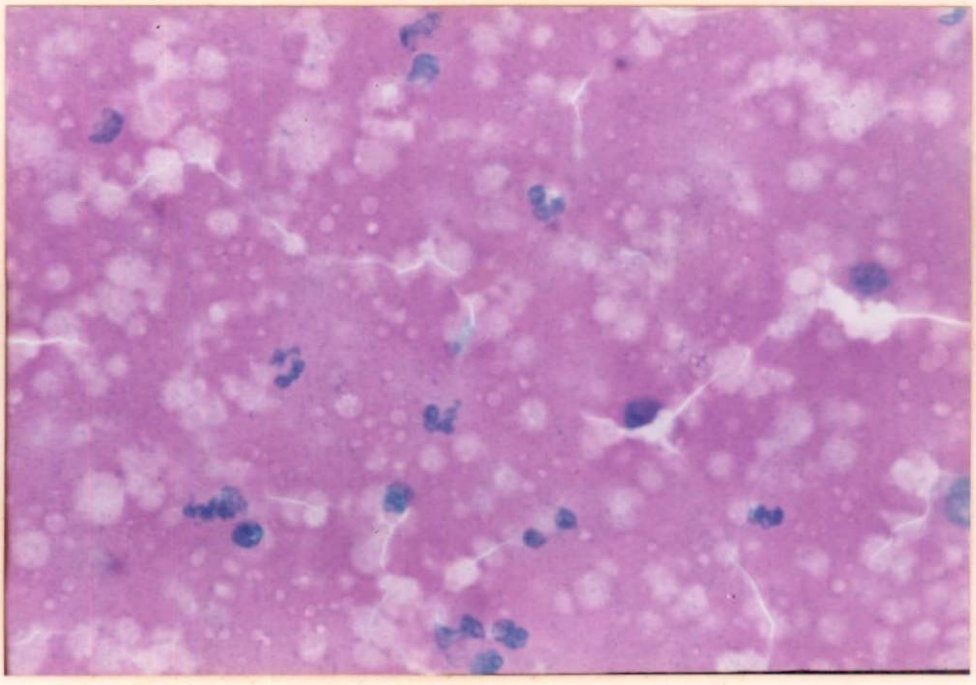


Fig.6 Coagulation of the rabbit plasma by coagulase positive Staphylococci

Fig.7 Growth of hemolytic organisms on blood agar

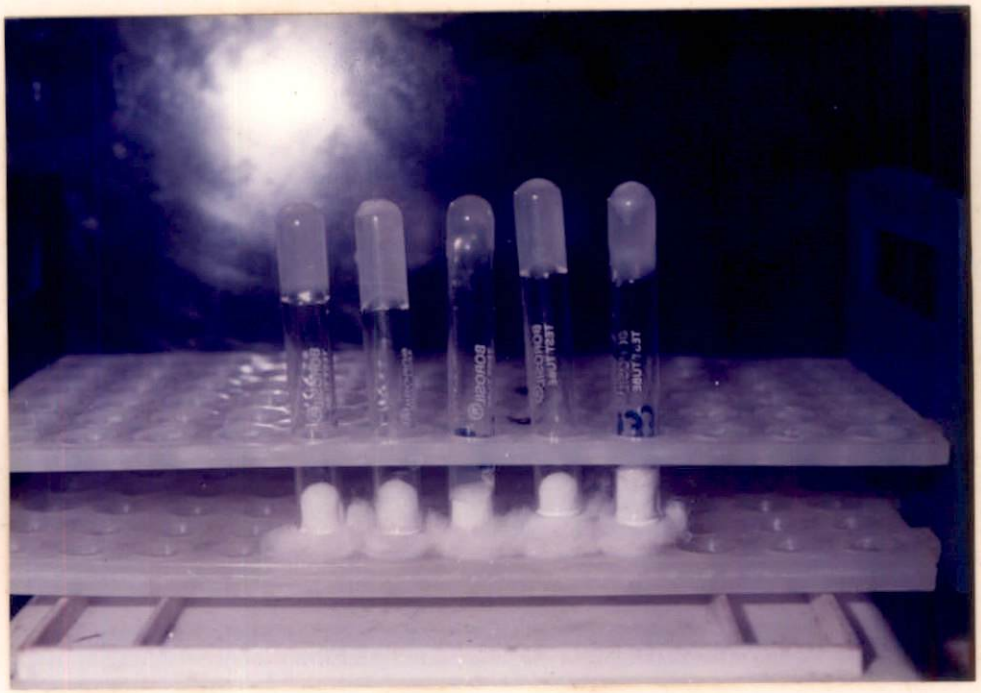
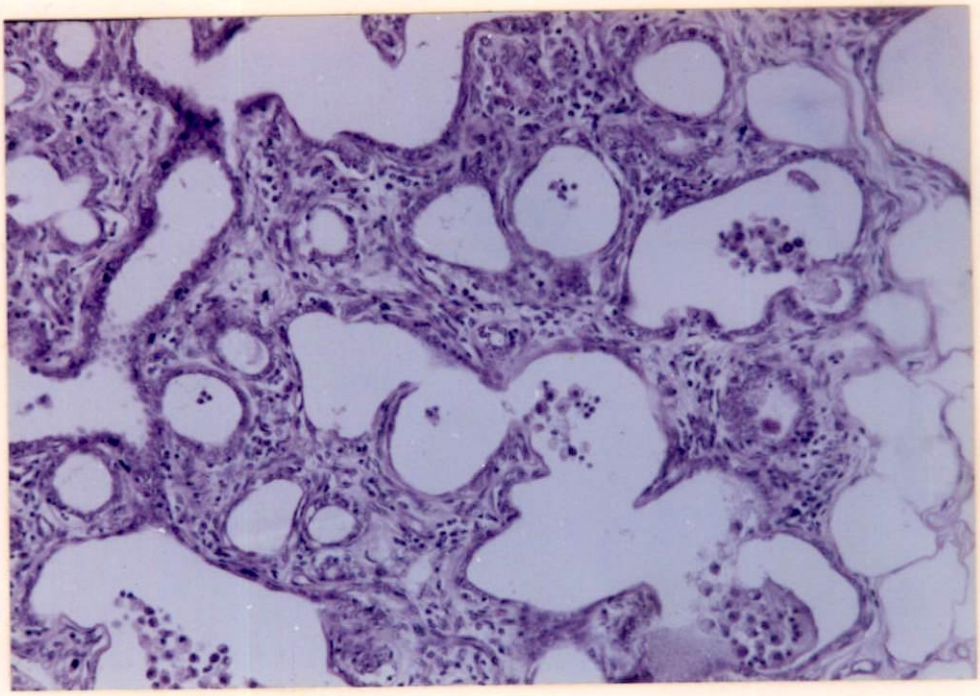


Fig.8 Infiltration of inflammatory cells into the acini of a involuting gland. H&Ex400

Fig.9 Various inflammatory cells in the acini of a lactating gland. H&Ex400



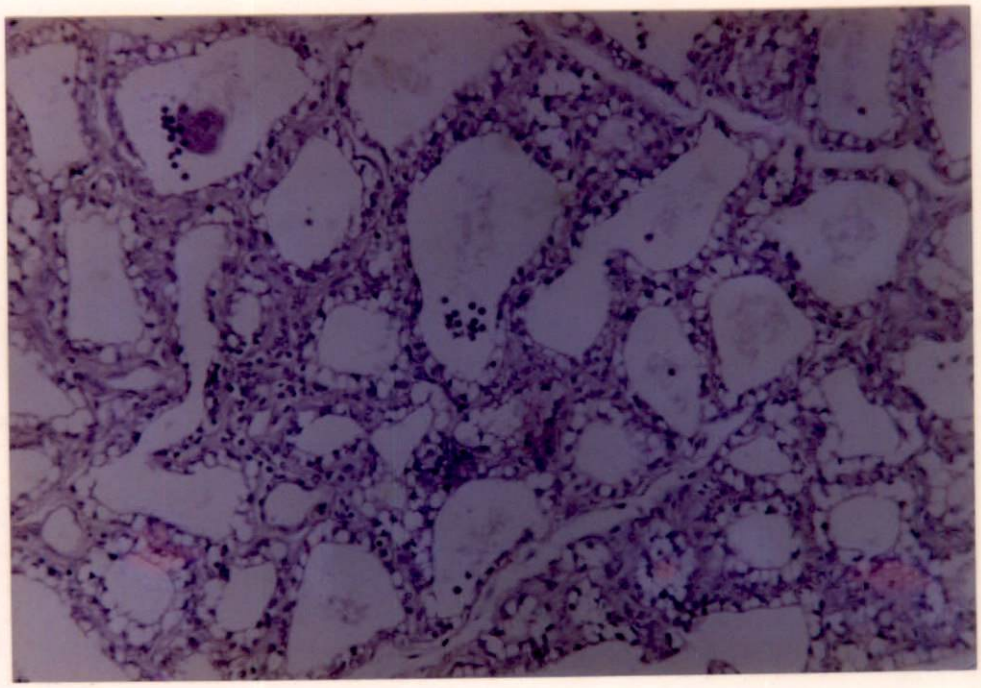
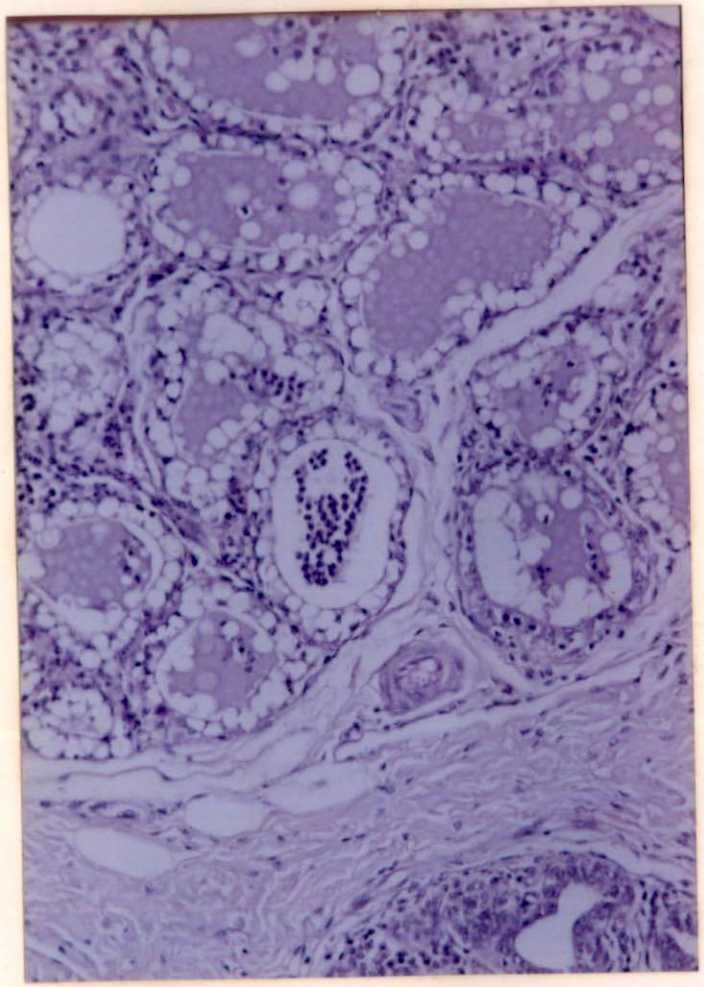


Fig.10 Aggregation of inflammatory cells in the acini of a lactating gland. H&Ex400

Fig.11 Occlusion of the acini by inflammatory exudates and cells. H&Ex400



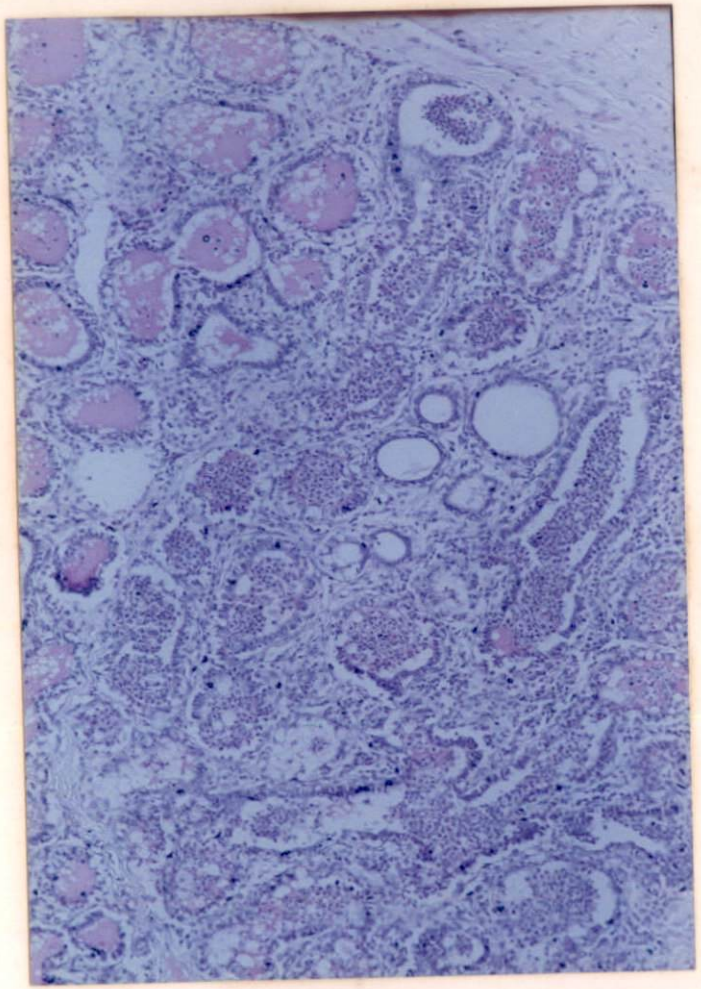
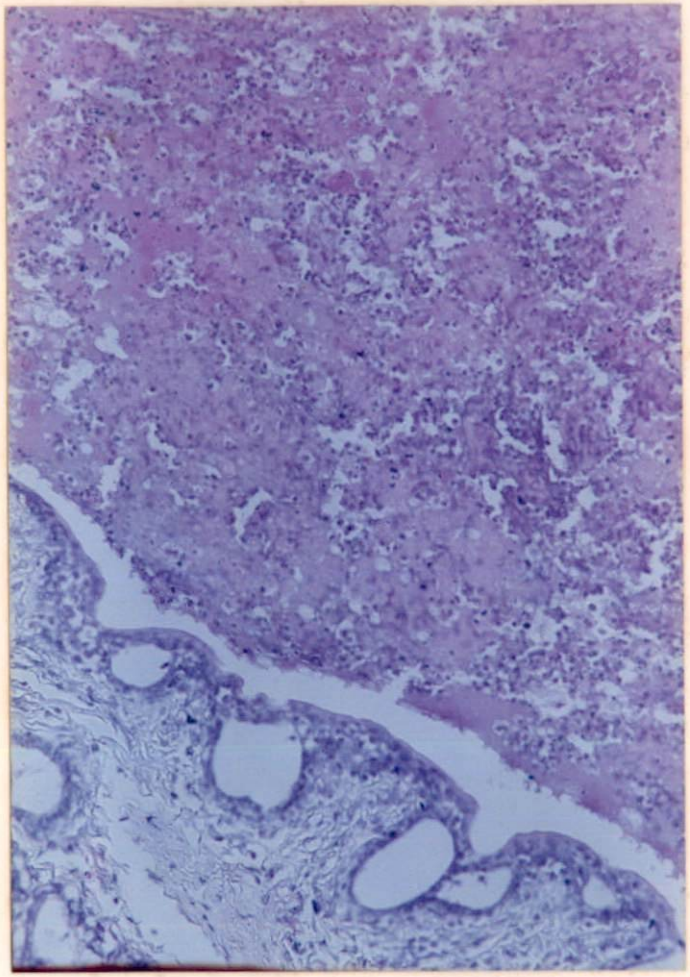


Fig.12 Inflammatory exudate and cells in a large lactiferous duct. Some of the inflammatory cells are necrosed. H&Ex400

Fig.13 Infiltration of the interstitium with inflammatory cells in a involuting gland. H&Ex250



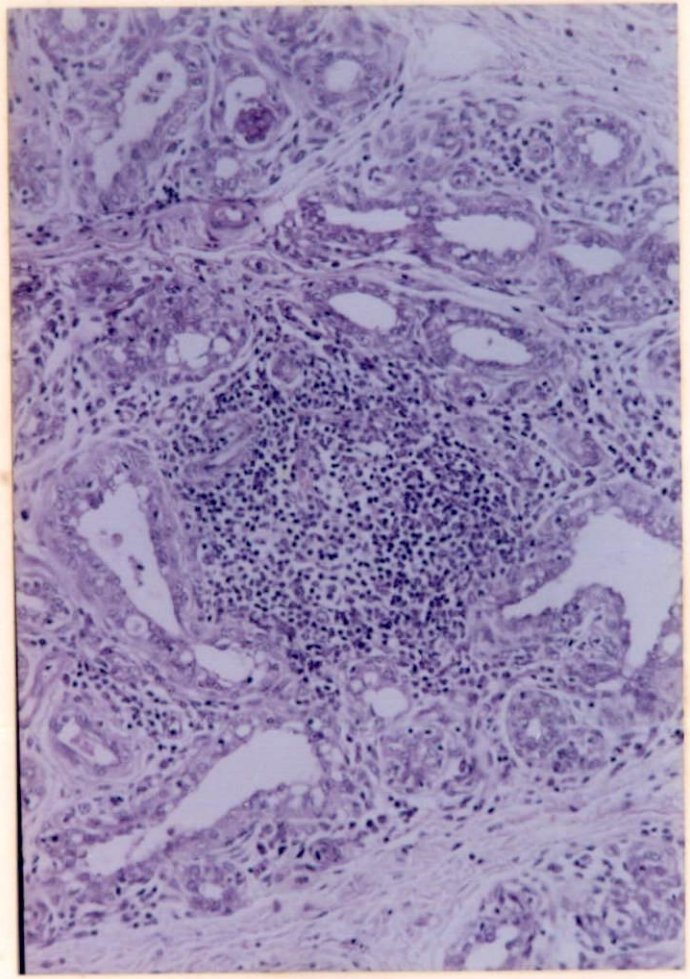
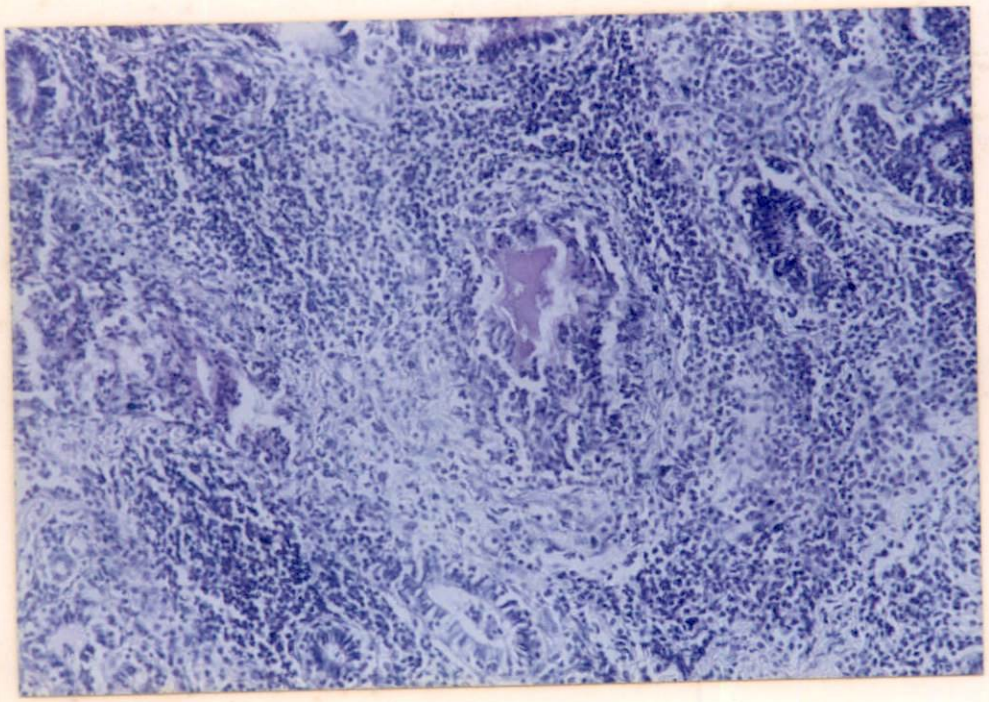


Fig.14 Destruction of the acini and some of the acinar lining cells in a lactating gland. H&Ex400

Fig.15 Intense infiltration of the interstitium of a lactating gland with inflammatory cells. H&Ex250



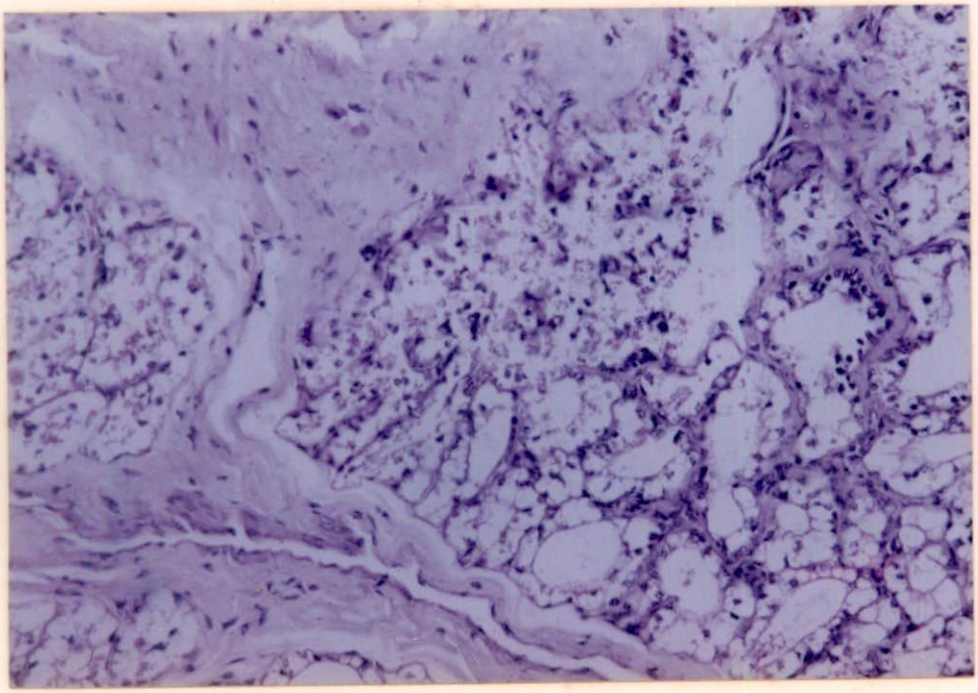
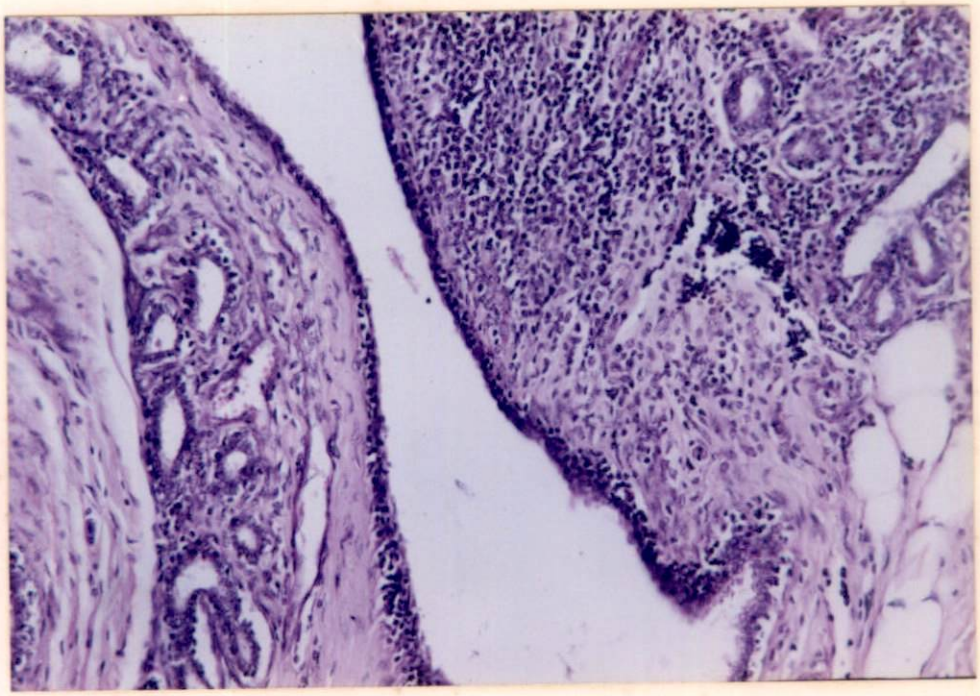


Fig.16 Infiltration of inflammatory cells around the inter lobular duct in a lactating gland. H&Ex250

ig.17 Infiltration and destruction of the interstitial area by inflammatory cells, in a lactating gland. H&Ex250



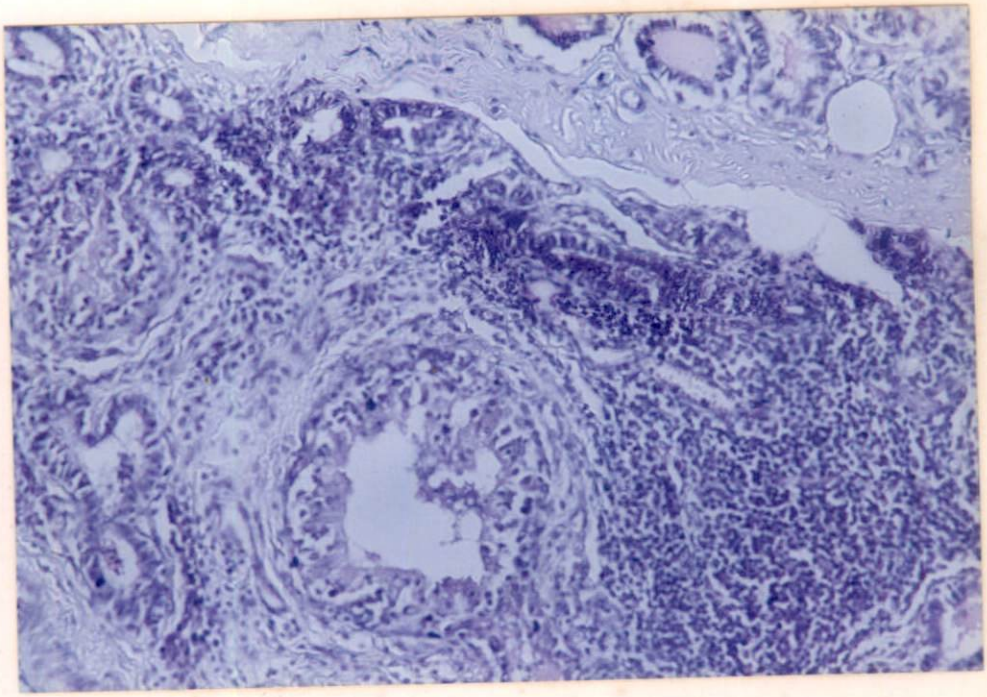
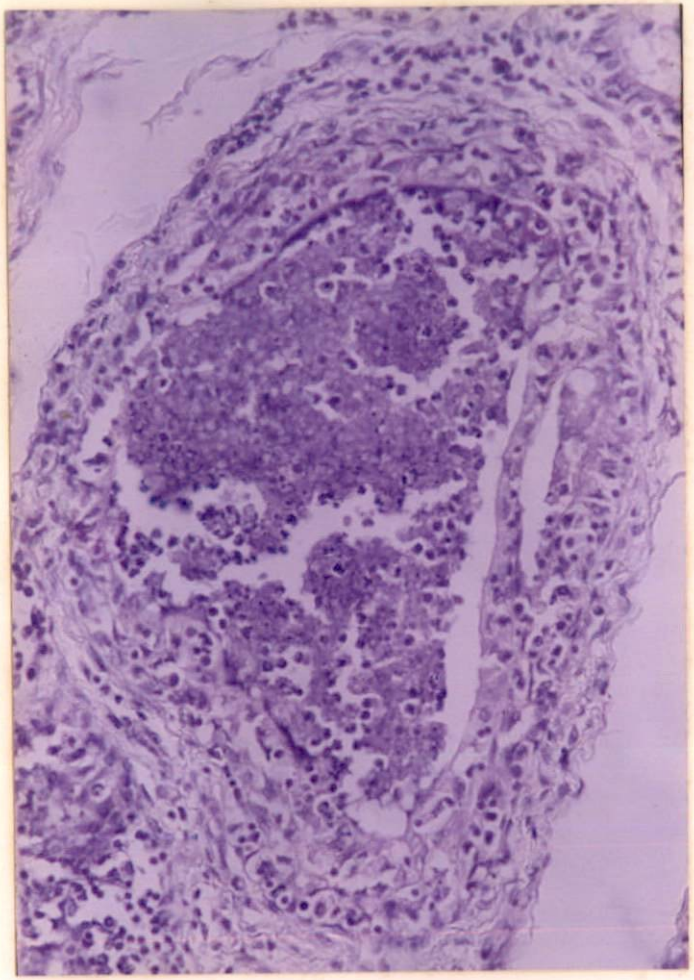


Fig.18 Non lactating gland showing infiltration of the large lactiferous duct with inflammatory cells. H&Ex250

Fig.19 Cortical area of lymphnode - Diffuse paracortical hyperplasia. H&Ex160



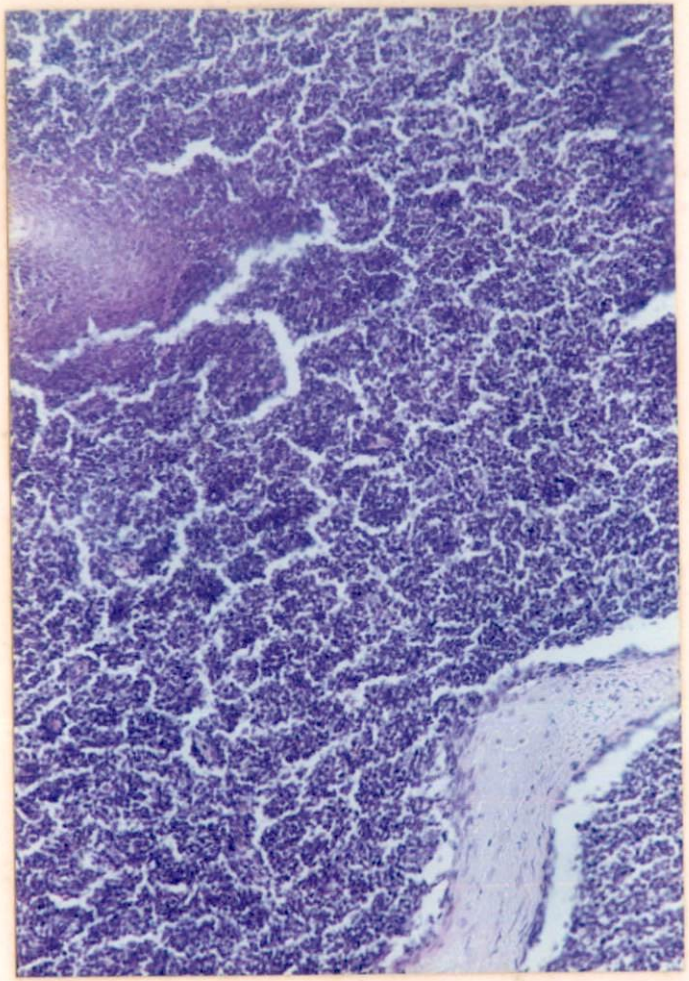
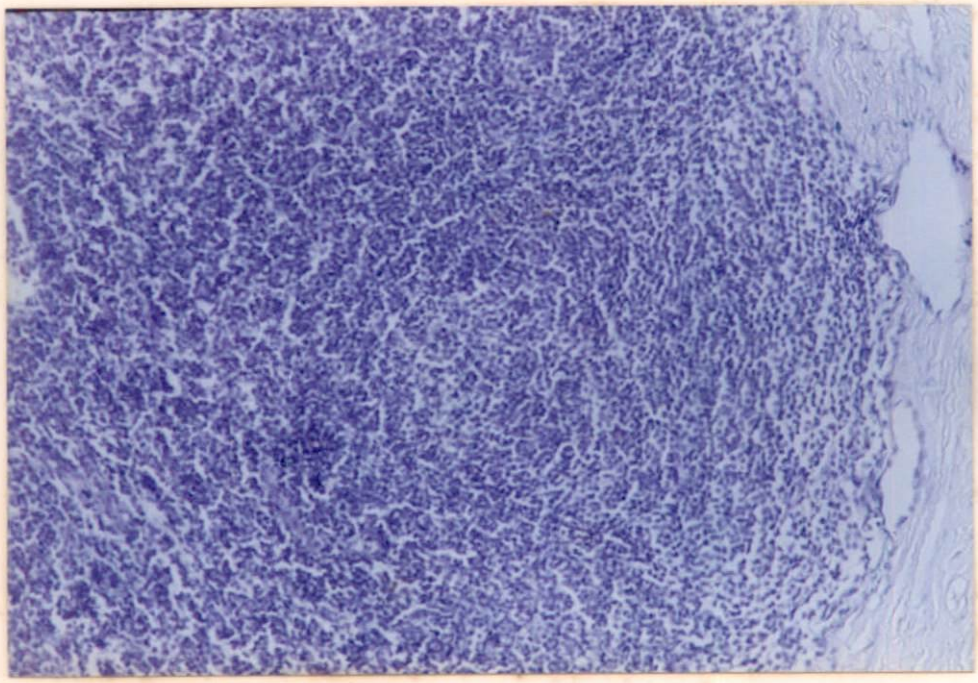


Fig.20 Medullary area of lymphnode - Diffuse lymphoid hyperplasia. H&Ex160

Fig.21 Cortical area of lymphnode - Lympho follicular hyperplasia. H&Ex250



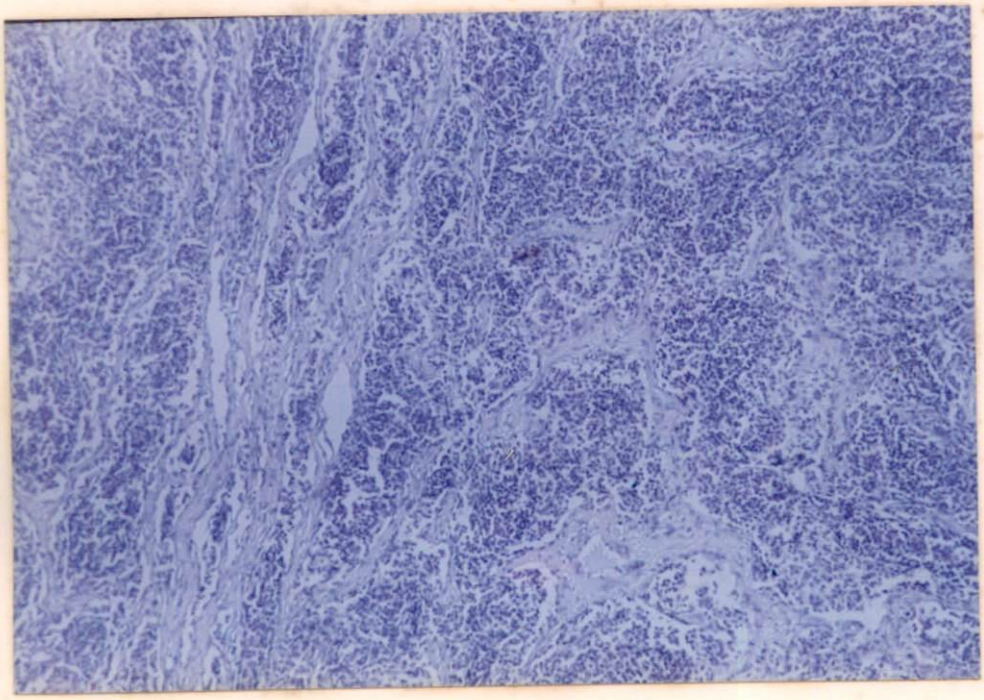
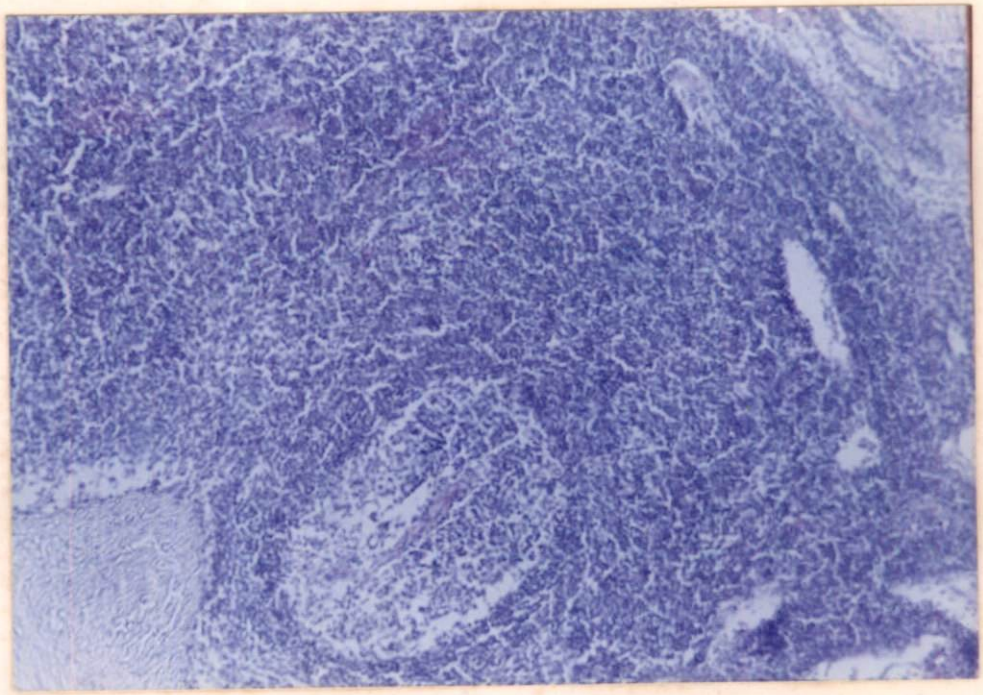
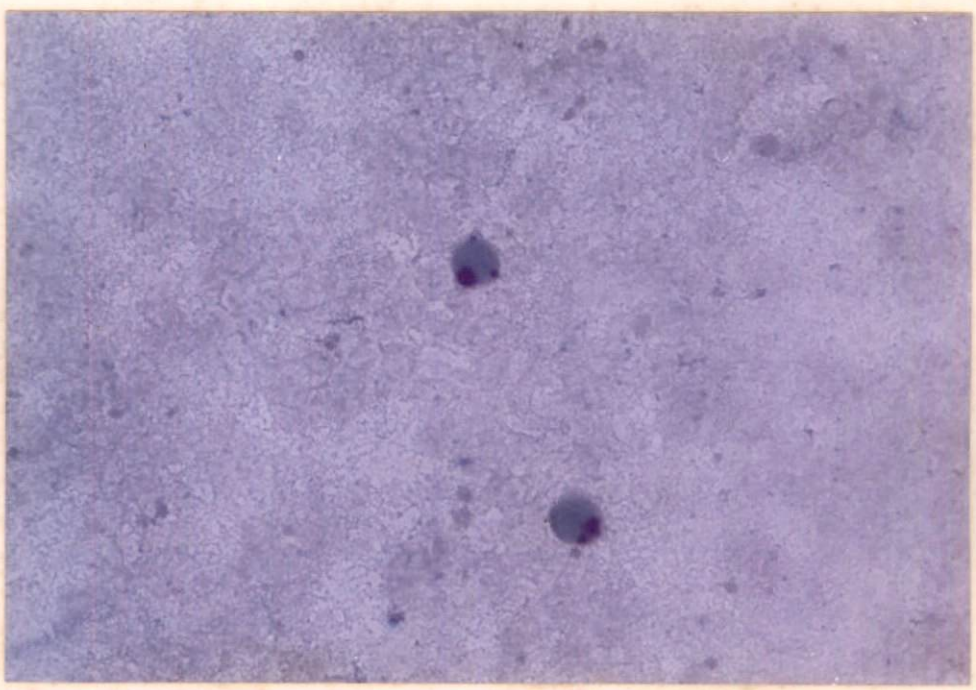


Fig.22 Cortical area of lymphnode - Paracortical lymphoid hyperplasia. H&Ex250

Fig.23 Alphanaphthyl Acetate Esterase (ANAE) positive lymphocyte in the peripheral blood smear x 1000





Discussion

DISCUSSION

In the present study out of the 227 milk samples tested for somatic cell count 89 samples showed cell count above 500000 cells with a mean value of 9,00,000 cells per millilitre of milk. This is very high compared with the cell count of normal milk which gave a mean cell count of 2,23,000 cells per ml of milk.

The presence of certain number of cells in milk has been universally accepted as normal. Several workers have shown that up to 500000 cells/ml of milk is the maximum for normal milk (Kehrli and Schuster, 1994). The number and types of cells in milk vary under physiologic and pathologic conditions. Epithelial cells are derived from the local tissue as a result of physiologic wear during milk secretion or as a result of tissue injury. Leukocytes are derived from blood. They infiltrate the mammary tissue and milk mainly in response to tissue injury irrespective of the cause. The neutrophil leukocyte count has been accepted as the best index of the presence and degree of inflammation in the bovine mammary gland (Blackburn and Macadem, 1954; Schalm and Lasmanis, 1963; Lalithakunjamma, 1976).

Dilution by milk influences the ultimate concentration of cells per unit volume of milk. So a mild reaction followed by

a low degree of leukocyte migration may not be noticed while doing the somatic cell count and California Mastitis test. Similar observation has been made by Fruganti and Valente (1980).

In mastitis diagnosis the cell count is of limited value unless used in conjunction with bacteriological tests (Schalm *et al.* 1971). In this investigation 101 samples out of the 205 samples examined, were positive for different types of bacteria. Comparison of somatic cell count and result of cultural examination showed that in majority of the infections with Staphylococci and Streptococci there was increased number of somatic cells. In the case of infections with Corynebacteria and Coliforms, there was a low somatic cell count in the milk samples. This was in accordance with the observations made by Schalm *et al.* (1971). Analysis of the somatic cell volume distribution can also be used as an aid to the diagnosis of mastitis (Hoarse *et al.* 1980).

The risk for clinical mastitis is always higher in those animals with low somatic cell count than in those with high count (Schukken *et al.* 1989). Milk somatic cells play a protective role against infectious disease in the bovine mammary gland. Many genetic and environmental factors affect the number and kinds of leukocytes that account for the vast majority of somatic cells in milk (Kehrli and Schuster, 1994).

The cell count together with determination of the neutrophil percentage was found to give a more reliable diagnosis of mammary inflammation than the California Mastitis Test. The cell count was considered particularly valuable to detect aseptic secretory changes due to mechanical injuries which might initiate an early stage of mastitis (Fruganti and Valante, 1980).

One hundred and fourteen samples out of the total 193 used for chloride test gave a positive result for the presence of chloride. During normal milk secretion, ions of sodium and chloride pass from the blood to milk accompanied by the resorption of water to maintain isotonisity. Since blood contains nearly four times the concentration of sodium chloride than milk, any condition bringing about altered permeability will raise the salt concentration of milk. The chloride has been taken as one measure of normal milk. Impaired lactose production is probably related to altered osmotic equilibrium induced by mastitis. Sodium chloride enters the milk from the blood as a result of altered permeability and raises the osmotic pressure of milk. Osmotic pressure of milk is brought into equilibrium with blood by a reduction in the secretion of lactose and is also accompanied by dilution of milk with more alkaline blood components, principally bicarbonates which increase the pH of milk (Schalm, 1977).

Chloride test is based on the principle that the increased amount of chloride in the milk reacts with the silver nitrate added to the milk and forms silver chloride to which an yellow colour is imparted by potassium chromate. When the chloride level is low, the silver nitrate added to the milk form silver chromate which imparts a reddish brown colour to the milk (Schalm *et al.* 1971).

In the present study California Mastitis Test, chloride test, somatic cell count and differential cell count were used for the diagnosis of sub-clinical mastitis. Emanuelson *et al.* (1987) have shown that combination of at least two diagnostic tests increases the predictability of mastitis. Direct correlation was observed between the California Mastitis Test score and somatic cell count, in this investigation.

Investigation on the various etiological agents causing mastitis showed that coagulase positive Staphylococci and coagulase negative Staphylococci were the major pathogens constituting 50% of the organisms, followed by Streptococci (41%), Coliforms (16%), Corynebacterium (12%), Pseudomonas (4%) and gram positive bacilli (2%). Similar observation has been made by many workers (Kapur and Singh, 1978; Dugalic *et al.* 1984; Chandra *et al.* 1989; Saxena *et al.* 1993; Singh *et al.* 1994; Reddy *et al.* 1994 and Tuteja and Kapur, 1995).

Antibiogram of the Staphylococci which included both coagulase positive and coagulase negative organisms showed that majority of them were sensitive to gentamycin (98.21%) followed by pefloxacin (96.42%), and chloramphenicol (91.07%). Similar observation has been made by Char *et al.* (1993) and Jha *et al.* (1994).

Streptococci were found to be equally sensitive to gentamycin and chloramphenicol (100%). Chloramphenicol has been reported as the most sensitive drug for Streptococci by Ramachandra *et al.* (1984). The present observation that chloramphenicol and gentamycin were the drug of choice for Streptococci was in accordance with those made by Sudharma *et al.* (1985).

Coliforms, the third major pathogen were more sensitive to gentamycin followed by chloramphenicol and pefloxacin. All the Coliforms were resistant to ampicillin and penicillin. Coliforms are widely distributed in nature and premises of dairy farms and the direct contact of teat with dung also enhance the chances of udder infection (Schalm *et al.* 1971). High humidity due to poor ventilation and over crowding increases the water content of the bedding material and this favours the existence of coliforms out side the body of the cow, especially when the cows lie down for a long time and the

body heat of the cow favours the multiplication of the organism outside the body (Jones, 1990).

Corynebacterium and Pseudomonas did not produce much increase in the cell count of milk. Similar observation has been made by Rainard (1987). Corynebacterium was found to give resistance against mammary infections either by non-specific immunomodulation or by bacterial antagonism.

Haematological evaluation revealed leukopaenia in acute and sub-clinical mastitis. This can be due to the movement of leukocytes en masse, from blood into the mammary gland and also due to the destruction of blood leukocytes by the endotoxin released as in coliform infections (Schalm *et al.*, 1971). The observations on the effect of endotoxin indicated that endotoxin leads to destruction of leukocytes existing in the milk and suggest that, as endotoxin is absorbed into the blood it may have a similar effect in reducing blood leukocyte numbers. This can be ascribed for the reduced somatic cell count in Coliform infection observed in the present study.

Packed cell volume was found decreased in sub-clinical and chronic stages of mastitis as compared to healthy non-mastitic cows. In acute cases only slight reduction in the mean value was noticed. Elevated packed cell volume was noticed in one of the acute cases and the etiological agent in this case was found to be coliform organism. All the other

animals in acute stage of mastitis gave more or less the same value as that of healthy non-mastitic cows. Schalm *et al.* (1971) observed an elevated packed cell volume in Coliform infections and explained that it might be due to the contraction of the spleen which forces sequestered erythrocytes into the blood stream.

Lymphocytes are the most important cells involved in the immune responses within the host. Elevated level of T lymphocytes was noticed in acute and chronic cases of mastitis as compared to normal healthy cows. Ishikawa and Shimizu (1983) noticed only a slight increase in the T lymphocyte count in cows with mastitis. The observation in the present study was in accordance with those by Kaura *et al.* (1989) in buffaloes where an increased number of T lymphocytes was seen in the peripheral blood of mastitis affected animals.

T lymphocytes were identified by their alphanaphthyl acetate esterase (ANAE) activity. By employing various methods the ANAE positive cells both in mice and human beings have been shown to be T cells by various workers (Muller *et al.* 1975; Knowles *et al.* 1978 and Pinkus *et al.* 1979). They compared the values obtained by E rosette forming technique and ANAE staining method and found that values were

almost similar and established that ANAE positive cells are E rosette forming T lymphocytes.

The T lymphocytes with one or two well defined circumscribed reddish spots in the cytoplasm were considered as positive cells. In the procedure described by Pinkus *et al.* (1979), the fixative and the staining solution are to be kept at a temperature of 4°C. In the present investigation, the ANAE activity was evaluated using the method described by Knowles *et al.* (1978). This differs from that of Pinkus in that it can be done at room temperature.

In the present study, the T lymphocyte count was 31.3 ± 1.42 per cent in healthy non-mastitic cows. This observation was almost similar to those made by Morein *et al.* (1979), who used Helix Promatia A (Haemagglutinin) as surface marker for T lymphocytes.

The presence of increased number of ANAE positive lymphocytes in the peripheral blood of mastitic animals when compared to the healthy non-mastitic cows were similar to the observation made by Anilkumar (1986) when he studied the immunopathological response of kids suffering from pneumonia.

The concentration of total protein and gammaglobulin in the serum was found to be almost same in healthy non mastitic cows as well as those in acute and chronic stages of mastitis.

A significant increase in the mean gamma globulin level was noticed in cows suffering from sub-clinical stage of mastitis.

Serum proteins pass directly from the blood into the milk. In the case of mastitis there will be change in permeability of the vascular system of the mammary gland, which has been proved by Dixon *et al.* (1961) using radioactively labelled serum immunoglobulins. The increased gammaglobulin level in the acute and chronic stage may be due to the increased stimulation of B lymphocytes, which produce the gammaglobulins. In sub-clinical stage there may not be sufficient passage of gammaglobulins from the serum, as there is no significant change in the vascular permeability of the gland. This may be the reason for the increased gammaglobulin seen in the present study.

Presence of large number of neutrophils in the acini and lactiferous ducts was the predominant sign in many of the lactating glands. Evidence suggests that the neutrophil leukocyte is involved in initiation of the inflammatory process in the bovine udder. Results of extensive studies on experimentally produced mastitis in neutropaenic cows have led to the conclusion that in the early phase of acute inflammation the neutrophil leukocyte is an important agent influencing magnitude and duration of the inflammatory response (Jain *et al.* 1972; Schalm, 1977).

Desquamated acinar lining cells were seen in a number of tissues. Some of the desquamated cells showed vacuolation in the cytoplasm. Such changes of the alveolar lining cells have been observed in the mammary gland during infections with *Staphylococcus aureus* (Gudding *et al.* 1984) Coliforms (Eberhart *et al.* 1979; Jones, 1990) and Streptococcus (Jubb *et al.* 1993).

Presence of lymphocytes and plasma cells along with epithelial cells and neutrophils were observed in few cases. This observation was in accordance with those made by Handique *et al.* (1988). They isolated *Staphylococcus epidermidis*, *Streptococcus uberis*, *Streptococcus bovis*, *Klebsiella aerogenes*, *E. coli* and Citrobactor spp. from such cases.

Involuted mammary glands were seen in the present investigation. Involution can be physiological or pathological. Streptococci were found to produce pathologic involution as well as fibrosis, as a result of acute exudative reaction. The first response to the invasion by of Streptococci is a remarkable interstitial oedema and extensive migration of neutrophils into the interlobular tissue and secretory acini. A few organisms persisted in the larger ducts and the neutrophil reaction was reduced, but macrophages and fibroblasts continued to increase in number and eventually obliterated many of the acini.

The involution affects lobules directly involved in the exudative inflammation as well as those surrounded by inter acinar tissue which contain fibroblasts: The process of fibrosis and involution continue until the end stages when some lobules show normally involuted tissue. Some are obliterated by fibrosis and others show a varying balance between both processes (Jubb *et al*. 1993).

Dialatation of the inter acinar and interlobular blood vessels was noticed in some tissues. In some other tissues there was extensive haemorrhage in the inter acinar area due to the damage to the blood vessels. Such changes have been noticed at the initial stage of mastitis (Beherens, 1984). The exotoxin or the endotoxin of the various etiological agents cause a change in the capillary permeability which is manifested by the dialatation of the vessels as well as escape of leukocytes, plasma and erythrocytes out of the blood vessels (Schalam *et al*. 1971; Gudding *et al*. 1984; Jones, 1990; Jubb *et al*. 1993).

In the case of infections with *Staphylococcus aureus* damaged epithelial lining has been noticed in the lactiferous ducts of some of the lactating glands. Gudding *et al*. (1984) observed early erosions and ulcer formation throughout the duct system. Replacement of such lining cells by

fibrocellular exudate was reported by Jubb *et al.* (1993) in the case of Coliform infections.

Few of the inflammatory cells in the acini and lactiferous ducts showed degenerative changes. This can be attributed to the phagocytosis of the bacteria by the leukocyte and the survival of the bacteria within the leukocyte leading to its destruction. Such observation has been made by Schalm (1977) in the case of infections with *Staphylococcus aureus* which produces alpha and beta toxins.

Corpora amylaceae were found in plenty in many of the tissues. They are named so because of their resemblance to starch grains, as they stain deeply with iodine. Corpora amylaceae are formed, when the desquamated acinar lining cells are compressed and neaded together tightly, which later undergoes hyalin degeneration. Their presence indicate an early inflammation in the mammary gland (Lalithakunjamma, 1976; Jones *et al.* 1990).

Some of the acinar lining cells were cuboidal in shape. Acinar lining cells can be columnar or cuboidal based on the stage of lactation. In an active gland the cells are tall and columnar or whereas in a gland that has stopped its secretion, the cells become short and flattened (Schalm *et al.* 1971).

Enlargement and oedema were the major changes noticed in the lymphnodes collected along with the inflammed glands. Inflammation of any organs causes similar changes in the associated lymphnodes. Eberhart *et al.* (1979) observed similar changes in the supramammary lymphnodes of cows affected with coliform mastitis. They also observed necrotic foci in the nodes.

The increase in the size of the cortical area and in the number of lymphoid follicles and germinal centres in the stimulated lymphnode can be considered as markers that indicate an active stimulation of the lymphnode.

Summary

SUMMARY

Immunopathological response of six cows each suffering from acute mastitis and sub-clinical mastitis and seven cows suffering from chronic mastitis was evaluated. The results were compared with ten apparently healthy non mastitic cows.

Elevated gamma globulin level was noticed in those having sub-clinical mastitis, showing and enhancement of the humoral immune response, whereas those suffering from clinical mastitis had only a slight increase in their gamma globulin level as compared to the healthy non-mastitic cows.

The T cell population was higher in acute (38.33 ± 0.8%) and chronic (35 ± 0.93%) stages of mastitis. The mean T cell population was 29.83 ± 1.54 per cent in the case of sub-clinically positive cows as compared to healthy non-mastitic cows in which the mean T cell count was 31.3 ± 1.42 per cent.

Diagnosis of sub-clinical mastitis was done using California Mastitis Test, chloride test, somatic cell count and differential count.

Clinically positive samples were also subjected to somatic cell count and differential count.

All the samples were also subjected to cultural and antibiotic sensitivity test.

Three hundred and thirty one samples were sub-clinically positive among the total 1489 milk samples examined. Elevated chloride level was noticed in all the sub-clinically positive cases.

Low somatic cell count was a feature of the culturally positive samples. Neutrophils were the major cells observed in milk, on differential cell count.

Staphylococci were the major pathogen detected on cultural examination followed by Streptococci, Coliforms, Corynebacterium, Pseudomonas, and gram positive bacilli.

Antibiogram of the various etiological agents proved that the major pathogens were more sensitive to gentamycin followed by pefloxacine and chloramphenecol.

Leucocytopaenia was a feature in the peripheral blood of cows suffering from sub-clinical mastitis. Slight reduction in the mean cell count was noticed also in case of acute mastitis.

Differential cell count showed neutrophilia in the case of clinically affected animals.

Serum protein level was normal in all the animals except in those having sub-clinical mastitis in which a slight increase was noticed.

Inflammatory reactions of varying intensity was noticed in 271 samples out of the total 336 samples examined, which included both lactating glands and those in various stages of involution. Acini and interstitium were infiltrated with the inflammatory cells in the case of lactating glands. Increased amount of fibroblasts were seen in many cases with focal collection of polymorphonuclear cells.

Oedema was the predominant lesion of the lymphnodes collected along with the inflammed mammary glands. Numerous lymphoid follicles leading to expansion of the cortex was seen microscopically in many of the lymphnodes.

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AN ASSESSMENT OF THE IMMUNOPATHOLOGICAL RESPONSE IN BOVINE MASTITIS

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ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences

KERALA AGRICULTURAL UNIVERSITY

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KERALA

1996

ABSTRACT

The immunopathological response of cows suffering from clinical and sub-clinical mastitis was evaluated and compared with healthy non-mastitic cows using various immunological markers.

Significant enhancement of the humoral immune response was noticed in sub-clinical cases of mastitis, whereas those suffering from clinical mastitis had only a slight difference as compared to the healthy cows.

Elevated T lymphocyte count was noticed in acute (38.33 ± 0.8%) and chronic (35 ± 0.93%) stages of mastitis. Those suffering from sub-clinical cases had a slight reduction in the mean T cell count, (29.83%) as compared to that of the healthy non-mastitic cows (31.3 ± 1.42%).

Three hundred and thirty one samples (22.23%) were sub-clinically positive out of the 1489 samples examined by California Mastitis Test. Out of the 520 samples subjected for cultural examination 186 were culturally positive. Fifty per cent of the culturally positive samples contained Staphylococci, which included both coagulase positive and coagulase negative group. Other pathogenic organisms isolated included Streptococci, Corynebacterium, Pseudomonas, and gram

positive bacilli. Direct correlation between the somatic cell count and California Mastitis Test score was noticed. Neutrophils were the predominant cells in the milk samples.

The major pathogens were more sensitive to Gentamicin (98.21%) followed by Pefloxacine (96.42%), Chloramphenecol (91.07%), Streptomycin (82.14%), Cotrimoxazole (80.35%), Ampicillin (73.21%), Oxytetracycline (71.43%) and Penicillin (60.71%).

Haematological examination of sub-clinical cases revealed leukopaenia. Slight increase in the serum total protein was noticed in sub-clinical cases.

Gross changes of mastitis was noticed in 18 mammary glands tissues examined. Histologically out of the 336 glands examined, 271 were having inflammatory changes of varying degrees. Acini as well as interstitium contained inflammatory cells in 37.68 per cent of cases. The entire parenchyma was affected in 14.49 per cent a cases.

Oedematous appearance of the supramammary lymphnodes was noticed. Microscopically marked expansion of the cortex by numerous lymphoid follicles in various stages of activity was noticed.