PREVALENCE OF YEAST AND YEAST LIKE FUNGI IN BOVINE MASTITIS AND THEIR *IN VITRO* DRUG SENSITIVITY

By

K. SUKUMAR

THESIS

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1996

DECLARATION

I hereby declare that this thesis entitled **Prevalence of yeast and yeast like fungi in bovine mastitis and their *in vitro* drug sensitivity** is a bonafide record of research work done by me during the course of research and that this 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

K Sukumar

Place Mannuthy
Date 23 -09-1996

K SUKUMAR

CERTIFICATE

Certified that this thesis entitled **Prevalence of yeast and yeast like fungi in bovine mastitis and their *in vitro* drug sensitivity** is a record of research work done by Sri K SUKUMAR 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

Place Mannuthy
Dated 23 09 1996

Dr P C JAMES
(Chairman Advisory Committee)
Professor
Department of Microbiology
College of Veterinary and
Animal Sciences

CERTIFICATE

We the undersigned members of the Advisory Committee of Sri K SUKUMAR a candidate for the Degree of Master of Veterinary Science in Microbiology agree that this thesis entitled **Prevalence of yeast and yeast like fungi in bovine mastitis and their *in vitro* drug sensitivity** may be submitted by Sri K SUKUMAR in partial fulfilment of the requirement for the Degree

Dr P C JAMES
(Chairman Advisory Committee)
Professor
Department of Microbiology
College of Veterinary and Animal Sciences
Kerala Agricultural University
Mannuthy Thrissur

Dr S SULOCHANA
Professor & Head
Dept of Microbiology
(Member Advisory Committee)

Dr G KRISHNAN NAIR
Associate Professor
Dept of Microbiology
(Member Advisory Committee)

DR C B MANOMOHAN
Associate Professor
Dept of Pathology
(Member Advisory Committee)

EXTERNAL EXAMINER

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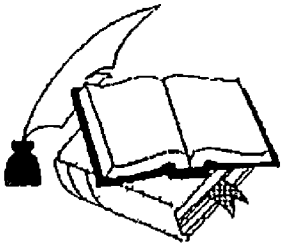
K SUKUMAR



Dedicated to
my Parents and Brother

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Introduction

INTRODUCTION

According to FAO statistics on Livestock (1988) the total population of cattle and buffalo in India were 201 4 million and 75 6 million respectively and these numbers constituted 14 per cent of the world s cattle and 50 per cent of the world s buffalo population During 1987 cattle alone produced 42 per cent (13 8 MMT) of the total milk produced (32 75 MMT) According to the annual report of the Department of Agriculture and Co operation, during the year 1987 88 the country produced 46 1 MMT which exceeded the target of 45 9 MMT Thus in milk production India stands first among the developing countries and third in the world

The gross value of output of milk and milk products amounts to over Rs 7 500 crores The average yield per cow and buffalo were 173 kg and 500 kg respectively This is very low when compared to the annual production per cow (3000-4000 kg) in countries where dairying has been well advanced

Improvement of indigenous breeds by selective breeding better feeding and management have helped to increase the average yield per cow However livestock industry is facing various disease problems One such malady is mastitis

In India, mastitis causes great economic loss to thedairy industry and is an obstacle in the achievemen s of White Revolution The new infections in the udder during the dry period can cause 35 per cent reduction in the milk yield in the affected quarters during next lactation (Blood *et al* 1983) According to an estimate by singh and singh (1994) there was an annual loss of about Rs 1607 20 crores due to mastitis in cow / buffaloes in India Financial losses are due to reduced milk secretion poor quality treatment cost and shortened productive life Hence control of mastitis is a very important aspect of health coverage to be taken care of

Mastitis is a disease complex of different aetiology and different degrees of intensity along with variations in duration and residual effects (Schalm *et al* 1971) The causative agents of mastitis may contaminate the milk from affected cows and may render it unsuitable for human consumption The various agents which cause clinical and subclinical mastitis may be hazardous to human health Prevention of mastitis will ensure the supply of wholesome and safe milk to the public

Bacteria are the predominant causative agents of bovine mastitis but presently fungi especially yeast and yeast like fungal organisms are also being recognised as primary agents for this condition Now a days mastitis is treated empirically and generally no attempt is being made to identify the causative agent and to determine its antibiotic sensitivity It is a well known fact that indiscriminate use of antibiotics for mastitis and other bacterial diseases has resulted in the emergence of antibiotic resistant strains (WHO 1978) The use of antibiotics as the standard treatment of bovine mastitis paves way for fungal mastitis (Ainsworth and Austwick 1973)

Though enough studies on the bacterial organisms associated with mastitis are available the reports regarding the isolation identificaion and antibiogram of the fungal agents associated with mastitis are scanty

Umpteen reports are available now with regard to the efficacy of essential oil/medicinal plant extract to combat fungal organisms causing different ailments in divergent species of animals

The *in vitro* antifungal sensitivity test will help to select the suitable antifungal agent for a particular fungal organism In this context for effective therapy of fungal mastitis it is necessary to identify and study the antibiotic sensitivity pattern of the agent responsible

In the light of the above facts it was decided to undertake a work to elucidate the following

i) Isolation of mycotic agents from milk samples of cows suffering from clinical mastitis

ii) Characterization and identification of the fungal isolates

iii) Drug sensitivity studies of yeast and yeast like fungi isolated using antifungal agents available in the market such as Amphotericin B Clotrimazole Fluconazole Griseofulvin Itraconazole Ketoconazole Nystatin and Pimaricin

iv) Extraction of essential oils from Cinnamon leaves and Clove and alkaloids of *Cassia alata*

v) Sensitivity of isolates to the extracts of plant origin mentioned supra

Review of Literature

REVIEW OF LITERATURE

Mastitis is one of the major disease problems in dairy herds causing heavy economic loss to the dairy industry

I Incidence of mycotic mastitis

Bacteria are the predominant causative agents of bovine mastitis but presently fungi especially yeast and yeast like fungal organisms are also being recognised as primary agent for this condition A variety of fungi were thought to play a role in bovine mastitis

Isolation of yeast like fungi from bovine milk was reported as early as 1901 Between 1934 and early 1950 s seven species of yeast were isolated from cases of bovine mastitis (Murphy and Drake 1947) During the 1950 s and 1960 s reports of yeast induced mastitis and identification of the involved species became much more common (Clarke 1960)

Stuart (1951) reported an outbreak of acute bovine mastitis following the infusion of udder with penicillin Yeast belonging to the genus Candida was isolated from such cases

Most significant findings in relation to the epidemiology of mycotic mastitis has been studied by Emmons (1955) Contamination of dry stored teat cups by a candida species was reported by Simon and Hall (1955) during an investigation of an outbreak of mastitis apparently caused by the same fungus

Trauma of udder improper and incomplete milking improper use of milking machines abnormalities of teat and udder improper feeding high milk yield early

lactation hereditory factors old age unsanitary conditions and poor management were said to be predisposing factors for bovine fungal mastitis (Henning 1956)

Trauma from the milking machine and use of irritating teat dips might predispose glands to yeast infection (Giesecke *et al* 1968) Yeast might be disseminated by certain species of birds (Mantovani *et al* 1970)

Yeast were saprophytic on plant and plant products and were normal inhabitants of skin of the udder and teats where they exist in low numbers (Loftsgard and Linquist 1960)

In India the first report of isolation of fungi from mastitis case was made by Dhanda and Sethi (1962) who recorded 0 2 per cent of cases of bovine mastitis due to yeast but they did not attempt to identify the fungal isolate

Prasad and Prasad (1966) reported cases of mycotic mastitis following intramammary antibiotic therapy Monga and Kalra (1971) examined 1204 milk samples from apparently healthy udders and clinically affected quarters of cows buffaloes and goats They could isolate fungus from 6 7 and 3 7 per cent of clinically affected quarters and 0 9 and 5 0 per cent of apparently healthy udders in the cases of cattle and buffaloes respectively

A study of the prevalence of yeast like fungi in the mammary glands of dairy cattle was conducted by Farnsworth and Sorensen (1972) in Minnesota Quarter samples from 6020 cows were cultured for yeast Growth of organisms was obtained from 3 2 per cent of the quarter milk samples The rate of yeast infection for Minnesota dairy cattle in this study was 2 0 per cent

Batches of contaminated multidose injection products used during the non lactating period were especially dangerous because lack of removal of the secretion allows yeasts to colonize in the gland more easily (Sipka and Petrovic 1975)

Sharma and Rai (1977) conducted a survey of subclinical mastitis in Uttarpradesh and reported an average incidence of 29 34 per cent in cattle and 20 89 per cent in buffaloes

Sharma *et al* (1977) conducted a survey to find out the incidence of fungal mastitis in clinical and subclinical form in cows and buffaloes They isolated fungi from 4 35 and 8 50 per cent of milk samples from clinical cases of mastitis in cows and buffaloes respectively In subclinical cases the values were 6 13 per cent in cows and 9 09 per cent in buffaloes

In a study in Egypt Awad *et al* (1980) examined 530 milk samples from cows and buffaloes They isolated yeasts from 6 1 and 6 5 per cent cases in cattle and buffaloes respectively

The incidence of mycotic mastitis appears to be on the increase because of extensive and rather indiscriminate use of antibiotics for treatment of mastitis (Gupta *et al* 1981)

Sharma (1983) in an attempt to find out the seasonal influence of fungal mastitis reported that the prevalence of mycotic mastitis was highest in winter and lowest in summer

Members of Cryptococcus genus were part of the normal resident flora of skin and digestive tract (Kirk and Bartlett 1986) Another important genus Trichosporon was common in air soil body surfaces and stagnant waters (Kirk and Bartlett 1986)

In another study out of 135 positive cases of mastitis seven (5 2%) were found to have been caused by fungi and 28 (20%) were identified to be mixed infection of fungi yeast as well as bacteria (Misra and Panda 1986)

Simaria and Dholakia (1986) examined 588 milk samples from the quarters of 150 apparently healthy cows Fungi were isolated from 44 samples of 34 cows From 82 milk samples from mastitis cases 24 cultures were isolated Thus the over all quarter wise incidence was 7 48 per cent in normal animals where as 29 27 per cent of clinical milk samples yielded fungi

In a study on 161 cows with mastitis in dairy farms in three districts of Bogor Indonesia during 1982 1984 the condition was chronic in 121 (75%) 137 (85%) had been treated with antibiotics unsuccessfully Out of 344 quarter milk samples cultured 116 (34%) yielded fungal agents mostly yeasts (Sudarwanto 1987)

Fungal agents were isolated from milk samples from 106 cows in Bikarner India by Mehrotra and Rawat (1989) Fiftyfive of these samples were taken from cows suffering from mastitis and the remaining from clinically healthy animals Thirty one and 51 fungal isolates were obtained from 27 mastitic and 26 normal samples respectively

Marcos *et al* (1990) in their studies on mycotic mastitis in cows and buffaloes isolated different species of fungi and the rate of isolation of different fungi ranged from 5 7 per cent to 33 per cent

Milk samples from 270 cows and 105 buffaloes with mastitis were screened for fungal infection by Singh *et al* (1992) They recorded that 23 (8 51%) and six (5 71%) were positive respectively Out of 1069 milk samples from affected quarters fungi were isolated from 5 87 and 2 09 per cent respectively

Sreeramulu *et al* (1992) reported that out of 110 milk samples collected from buffaloes with clinical mastitis 11 81 per cent were positive for fungi and 17 per cent positive both for bacteria and fungi They also observed that the incidence of mycotic mastitis increased with age and also with parity

In a survey of 2078 milk samples from normal clinical and subclinical mastitis from 22 dairy herds of 16 districts in the state of Sao paulo Brazil fungi were isolated from 12 07 per cent samples (Costa *et al* 1993)

Aalbaek *et al* (1994) examined mammary secretions (n – 2896) from Danish cattle for one year period with clinical or subclinical mastitis and recovered 45 strains of fungi and algae

Out of 800 milk samples screened by Daljeet Chhabra *et al* (1996) fungi were isolated from 137 cases The over all percentage of infection was found to be more in buffaloes (28 66%) than in cows (21%)

2 Aetiology and identification

Different species of fungi were isolated by different workers from clinical as well as subclinical mastitis cases Isolation of fungi from milk was reported as early as 1901 (Farnsworth 1977)

Stuart (1951) isolated Candida spp from an outbreak of acute mastitis in Weybridge Steele Bodger (1953) screened seventeen cases of mastitis in lactating cows during the period of a few months Out of 17 cases 10 yielded pure culture of yeast four were mixed infections of *streptococcus agalactaiae* and yeast infection and three were mixed infections of staphylococcus and yeast infections Ainsworth and Austwick (1959)

have described 26 species of fungi associated with primary and secondary mycotic mastitis

Overgoor (1960) reported isolation of *Candida albicans* from mastitis cases from Netherland

Heidrich and Renk (1967) and Jubb and Kennedy (1970) considered more than 50 species of bacteria and 20 species of yeast like fungi being associated with mastitis in bovines

Bolck *et al* (1967) reported the isolation of C pseudotropicalis from cases of mastus in Germany Singh and Singh (1968) isolated *A terreus C tropicalis* and *C guillermondi* from bovine mastitis cases

Monga *et al* (1970) isolated five strains of *Cryptococcus neoformans* out of 432 milk samples from bovine mastitis This fungus had been reported from many human cases of CNS infection (Mohaptra 1969) Therefore this fungus in milk poses a danger to human health by the consumption and handling of raw milk

Monga and kalra (1971) isolated *C neoformans C albicans C krusei C parapsilosis* and Saccharomyces spp from clinical mastitis milk samples of cows Farnsworth and sorensen (1972) isolated *Candida krusei C Parakrusei C guillermondi and C tropicalis* from dairy cows

Jand and Dhillon (1975) isolated yeast and yeast like fungi *C parapsilosis C tropicalis C stellatoidea C guillermondi C albicans Cryptococcus alter Rhodotorula glutins* and *Geotrichum candidum* and moulds namely *Aspergillus fumigatus* Alternaria spp Rhizopus spp and Penicillium spp from the milk samples of cows buffaloes and goats

Davise Honig larone (1976) reported the identification of filamentous fungi (mould) based on their rate of growth general topography texture surface pigmentation, reverse pigmentation, and microscopic appearance

Sharma and Rai (1977) isolated *A terreus* Candida spp and Trichosporon spp from the bovine subclinical mastitis cases Thompson *et al* (1978) isolated *Petriellidium boydii* and *Aspergillus fumigatus* from the milk and mammary tissue of two different cows

In a study in Egypt, Awad *et al* (1980) examined 530 milk samples from cows and buffaloes They could isolate yeasts from 6 1 and 6 5 per cent of cases in cattle and buffaloes respectively The isolates were Candida, Torulopsis Trichosporon, Rhodotorula and Saccharomyces spp

Yousef Al Doory (1980) reported the identification of yeast and yeast like fungi based on their microscopic morphology growth on corn meal agar containing Tween 80 germ tube test urease test sugar fermentation and sugar assimilation test He also explained the animal inoculation technique for identification of fungi

Richard *et al* (1980) obtained 91 cultures of yeast from infected mammary glands of cows in New York and Iowa Of the isolates 78 per cent belonged to the genus Candida Eleven Candida spp were found with C tropicalis being the most frequently isolated species followed by C rugosa

Saito (1980) isolated Candida rugosa, *Saccharomyces bailli T cutaneum* and *C krusei* from cases of clinical mastitis

Aspergillus fumigatus was reported to cause mastitis in cows (Matsuoka 1981) A survey of 91 bovine cases of fungal mastitis in the USA showed that 78 per cent belonged to Candida spp

Douglas VanDamme (1983) used blood agar and phenol red mannitol agar for culture Yeast grew well on both agars but growth was more rapid on phenol red mannitol agar

Kadic *et al* (1983) examined 3849 samples of milk or udder secretion from 982 cows Out of this 108 yielded 110 yeast strains the commonest genus was Candida (8 species 93 strains) particularly *C guillermondii* (41 strains) and *C krusei* (18 strains) The remaining isolates included *Trichosporon cutaneum Pichia membranefaciens Debaryomyces hansenii* and Saccharomyces spp Pal and Mehrotra (1983) isolated *C neoformans* from two mastitis milk of a buffalo and a cow

Sharma (1983) isolated *C Albicans C tropicalis C krusei Cryptococcus spp A terreus A flavus Aspergillus fumigatus A niger Cladosporium olivaceum Trichosporon cutaneum* Rhizopus and Penicillium spp from bovine mastitis

Tanwani and Yadava (1983) reported the isolation of *C neoformans C albicans C parapsilosis C krusei* Geotrichum, Trichosporon, Blastomyces Aspergillus Mucor Rhizopus Actinomyces and Nocardia spp

Misra and Panda (1986) isolated *A flavus A niger A amstelodami A chevalieri A sydowi Geotrichum candidum* and Penicillium spp from 135 positive cases of mastitis in cows

Sakurai (1986) isolated 18 fungal strains from 158 quarters (72 cows) of clinical cases and 14 fungal isolates from 110 quarters (50 cows) of subclinical bovine mastitis The 18 fungal isolates from clinical cases comprised two strains of *C krusei* one of *C parapsilosis* six *Debaryomyces hansenii* three *Torulopsis glabrata* and four *Rhodotorula rubra* The 14 isolates from subclinical cases included one strain of *C tropicalis* two each of *C rugosa D hansenii* Torulopsis spp and one *Saccaharomyces cerevisiae*

Cuci and Matraku (1987) isolated Candida, Trichosporon, Rhodotorula Geotrichum Aspergillus Penicilhum, *Trichoderma viridae T conningii Trichotechium roseum* Alternaria Curvularia Paecilomyces and Cephalosporium spp from clinical and subclinical mastitis milk sample of cows

Dhabali Singh *et al* (1989) examined 375 lactatmg animals (270 cows and 105 buffaloes) Out of which 29 cases (23 cows and six buffaloes) were found suffering from mycotic mastitis The fungi isolated were *C albicans* (3) *C krusei* (3) *C parapsilosis* (6) Cladosporium (11) Geotrichum (4) Alternaria (5) Fusarium (2) Rhodotorula (1) Rhizopus (1) *Aspergillus fumigatus* (3) and *A niger* (11)

Mehrotra and Rawat (1989) isolated Aspergillus spp (from 12 mastitic and 3 normal samples) Cephalosporium spp (from 3 mastitic sample) Mucor spp (3 mastitic) Alternaria, Curvularia Penicillium and Rhizopus spp(1 mastitic and 1 normal sample each) Absidia and Monilia spp (1 normal sample each) and Helminthosporium spp (1 mastitis sample)

A method to detect fungi and algae m milk by means of fluorescence microscopy using fungiqual was described by Deutz and Kuttin (1990) They detected *C albicans* (2 cases) *C famata (Torulopsis candida)* (2) *C guillermondii* (2) *C humicola* (1) *C inconspicua* (3) *C krusei* (3) *C parapsilosis* (2) *C rugosa* (2) *C tropicalis* (1) *Rhodotorula rubra* (2) and *Trichosporon cutaneum* (1) in the 21 positive milk samples

Marcos *et al* (1990) isolated *Aspergillus fumigatus* (37 6% and 36%) *A niger* (14 6% and 26 4%) *A flavus* (11% and 17%) *A minor* (11% and 1ɔ 4%) *Candida albicans* (40 2% and 52 8%) *C tropicalis* (19 5% 24 5%) *C krusei* (14 6% and ɔ 8%) Mucor spp (33% and 28 6%) and Rhizopus spp (4 9% & 5 7%) from 210 Friesian cows and 182 buffaloes respectively

Cows and buffaloes with mastitis were screened for fungal infection by Singh *et al* (1992) showed that Candida spp accounted for 24 per cent of the cases (*C albicans* (3) *C Krusei* (3) and *C parapsilosis* (6)) Other fungi isolated were Cladosporium (11) *Aspergillus niger* (11) Alternaria, (5) Geotrichum,(4) *Aspergillus fumigatus* (3) Fusarium (2) and Rhodotorula and Rhizopus spp one each

The importance of identification of the causative agent with reference to prognosis was discussed by Veen ~~*et al*~~ and Kremer (1992) and they opined that cases due to Candida recovered spontaneously whereas in those due to Cryptococcus spontaneous recovery was rare

Costa *et al* (1993) isolated yeast of Crypotococcus spp (71 strains) Rhodotorula spp (40) Candida spp (68) *Trichosporon cutaneum (T beigelii)* (21) *Aureobasidium pullulans* (7) *Pichia ohmeri* (1) and *Geotrichum candidum* (1) The mould isolated were of the genera Aspergillus (3) Penicillium (3) Alternaria (3) Phoma (3) and Epicoccum (2)

Gosch (1993) recovered 122 isolates of yeasts and identified them as *Candida guillermondii*(28 isolates) *C krusei* (23) *C rugosa* (16) *C parapsilosis* (9) *C psuedo tropicalis* (8) with smaller numbers of five other Candida species and four isolates of *Pichia farinosa*

Kuo and Chang (1993) reported that of the 91 isolates from 76 (9 5) quarter milk samples of cow 58 (63 7%) were Candida comprising 15 *C tropicalis* 10 *C parapsilosis* nine *C rugosa* seven *C guillermondii* and s x *C albicans* Others were *Geotrichum candidum* (13) *Trichosporon beigelii* (10) *C humicola* (2) *C krusei* (2) *C lipolytica* (3) *C lusitaniae* (3) *C stellatoidea* (1) *Hansemula anomala* (1) *Saccharomyces cerevisiae* (1) *S rosei* (*Torulaspora delbrueckii*) (2) *Torulopsis candida* (1) *T inconspicua* (3) *T maris* (1) and *Trichosporon penicillatum* (1)

Aalbaek *et al* (1994) isolated *Candida catemdata* (2 isolates) *Candida kefyr* (6) *C krusei* (17) *C rugosa* (6) *C tropicalis* (3) *C valida* (1) and *Geotrichum capitatum* (*Blastoschizomyces capitatus*) (5) from 44 mammary secretions of 42 cows in 40 herds

Reddy and khan (1994) cultured 263 milk samples from cross bred cows with clinical mastitis and showed that 19 (7 2%) samples were positive for fungi *Aspergillus niger* (5 25 3%) was the commonest isolate followed by *Aspergillus fumigatus* (4 21 %) *A flavus* (3 16 %) Penicillium spp (3 16%) Fusarium spp (2 10 %) and two (10%) unidentified fungi

3 Characteristics of fungal mastitis in animals

A herd of 26 cows had been treated with mammary infusion of penicillin oil wax suspension daily for five days in a *Streptococcus agalactiae* eradication experiment in Middle Sex during 1948 Fifteen quarter of eleven cows developed an acute mastitis between the second and seventh days after the end of the treatment There was severe induration of the udders a marked drop in milk yield, febrile reaction of about 106°F and the udder secretions were watery with large yellow clots The mastitis lasted about a week without treatment and infusion of penicillin had no beneficial effect in few quarters Yeasts were isolated in pure culture from all the affected quarters (stuart, 1951)

Severity of the clinical mastitis appeared to be related to genus of the yeast involved

Candida spp Bovine mammary infections caused by Candida spp were often mild and transient some were self limiting More severe infections had followed antibiotic infusions Candida tropicalis was incriminated in one outbreak in which all treated animals developed acute mastitis with varying degree of severity The udders developed extensive swelling of a spongy consistency Milk from the affected quarters was grey and

viscid Body temperature ranged from 104°F to 107°F Some animals were anorectic and lame and milk production was drastically decreased (Loken *et al* 1959)

Candida maltosa mastitis was characterized by swelling and induration, it was progressive inspite of treatment with penicillin, oxytocin and intramammary infusion with streptomycin and nystatin (Kitamura *et al* 1990)

The clinical signs elicited by invasion of the bovine mammary gland by *Cryptococcus neoformans* were extremely variable. Cryptococcus infection caused severe clinical mastitis characterized by initial swelling of the quarter febrile spikes inappetance and severe loss of milk production, which continued indefinitely Mammary tissue became replaced by granulomatous tissue (Pounden *et al* 1952)

The mammary gland was markedly fibrous and numerous areas of abscessation were observed on cut section The hypertrophied supramammary lymph nodes measured 14 by 10 by 4 cm and were soft and edematous (Simon *et al* 1953)

Trichophyton caused severe local inflammation systemic involvement and severe decrease in milk production However majority of cows infected with this organism returned to normal in two to four weeks period with no residual effects Infection with this organism did not usually result in formation of excess scar tissue or persistent decrease in milk production (Stuart 1951)

Coccidioides immitis Caused swollen mammary gland unresponsive to antibiotic treatment fluid expressed from the gland was clear gelatinous and yellow intermixed with strands of cloudy material Mammary gland was shrunken and hard with yellow caseous nodules 3 8 mm in diameter (Walker *et al* 1993)

Aspergillus spp The *Aspergillus fumigatus* affected udder tissue showed many nodules up to five mm diameter which were granulomatus with yellow green caseous centres containing fungal hyphae (Thompson *et al* 1978)

In case of infection by *Aspergillus fumigatus* or *A nidulans* there were multiple abscesses in the quarters These were surrounded by granulation tissue but the milk ducts were unaffected (Thompson *et al* 1978)

The clinical development of the disease and its spread through out the quarters occurred in 12 days (Ferreiro *et al* 1989)

4 Antifungal susceptibility Test

In vitro antibiotic sensitivity testing by agar diffusion method is the mostly accepted procedure because of its simplicity and rapidity (Anderson, 1970)

a) Antifungal Chemotherapeutic agent

Kucharski and Rozewicka (1974) found sensitivity of Candida species to Mycostatin *In vitro* studies by Lipinicki *et al* (1975) also revealed the sensitivity of *C albicans C guillermondii C tropicalis C stellatoidea* and *C pseudotropicalis* to Mycostatin

Jand *et al* (1978) studied the inhibitory action of Mycostatin Griseofulvin and Thiobendole against *C albicans C tropicalis C stellatoidea C parapsilosis Candida guillermondii* Rhodotorula spp and Saccharomyces spp He found that saccharomyces spp was sensitive to Thiobendole o ily while Griseofulvin had no effect on the growth of any of these organisms

Tortorano *et al* (1979) tested Amphotericin B 5 fluorocytos ne Miconazole Econozole and Clotrimazole against 45 stra ns of *C albicans* 15 *C tropicalis* two

C guillermondii five *C parapsilosis* two *C zeylanoides* 12 *C pseudotropicalis* three *C krusei* seven *Torulopsis glabrata* 18 *C neoformans* two *Geotrichum candidum* and one *Saccharomyces cerevisiae*

Saito *et al* (1980) revealed that pyrrolnitrin, clotrimazole amphotericin B and pimaricin were relatively useful for mycotic mastitis

Zaror *et al* (1981) tested 100 fungal isolates against Clotrimazole All were susceptible to Clotrimazole at 16 µg/ml and 99 per cent were susceptible to 8 µg/ml or less

Hartmann and Kilchsperger (1982) studied 19 Candida strains isolated from milk All the strains were inhibited by natamycin at or above 6 25 µg/ml

Minagawa *et al* (1982) reported that the ketoconazole had minimum inhibitory values (MIC) of 71 6 µg/ml against *C albicans* 0 62 0 80 µg/ml against other Candida spp 1 76 7 94 µg/ml against other yeasts 0 63 20 µg/ml against dermatophytes 1 25 20 µg/ml against Aspergillus spp 1 25 2 5µg/ml against dematiaceous fungi 8 41 µg/ml against *Sporothrix schenckii* 0 62 5 µg/ml against Trichosporon spp and 80 µg/ml against *Fusarium solani*

Yeo and Choi (1982) studied the various antifungal agents against different fungi The MIC of clotrimazole was 12 5 µg/ml for all isolates except one *C albicans* isolate

Mahajan (1986) tested the *in vitro* susceptibility of 18 fungi to natamycin econazole ketoconazole and amphotericin B None of the fungi were inhibited at 0 5 0 µg/ml of natamycin 13 were inhibited at 10 µg/ml Econazole arrested the growth of seven fungi at a concentration of 0 5 µg/ml Hundred µg/ml of ketoconazole produced a

similar effect amphotericin B even at a concentration as high as 100 µg/ml inhibited only few fungal species

In vitro sensitivity trials of Yeast and mould isolates from bovine udder by Shah *et al* (1986) revealed high efficacy of nystatin to inhibit most of the isolates Actidione was found to have effect only on yeast cultures only while thiobendole had no effect on the growth of any of the isolates

Nerson *et al* (1987) reported a standard antifungigram technique used by seven laboratories for determination of the sensitivity of 476 yeast strains and 28 isolates of filamentous fungi to seven antifungal agents at two concentrations Ticonazole showed the best *in vitro* activity against yeasts followed by ketoconazole amphotericin B and other imidazoles

Steiman *et al* (1988) studied the sensitivity of *M gypseum* to five antifungal agents by disc diffusion method The sensitivity of *M canis M gypseum* and *T interdigitale* to five antifungal agents indicated the efficacy of imidazole compounds as compared to amphotericin B and nystatin

Bansal *et al* (1989) tested the antifungal sensitivity of three candida spp (*C krusei C guillermondii* and *C parapsilosis*) isolated from clinical mastitis of cattle and buffaloes to five antimycotic drugs Clotrimazole (5 µg/ml) ketoconazole (20 µg/ml) miconazole (20 µg/ml) and nystatin (80 units/ml)were found to completely inhibit the growth of Candida spp Griseofulvin (25 µg/ml) did not prevent the growth of any Candida spp Sensitivity testing by disc diffusion method revealed that clotrimazole (5 µg/disc) ketoconazole (10 µg/disc) and miconazole (25 µg/disc)were highly effective drugs with the exception of *C guillermondii* which was moderately sensitive to miconazole at this concentration

Katamoto and Shimada (1990) treated bovine mycotic mastitis case by combined intra arterial and intramammary injection of miconazole on three successive days

Blanco *et al* (1992) studied the *In vitro* activities of some antifungal agents against *Candida albicans* ATCC 10231 by turbidimetric method The drug concentration which inhibited 50 per cent of growth (IC 1/2) the lowest drug concentration at which the growth was just <30 per cent of that in a positive control well (IC 30) the visual inhibitory concentration (ICV) and minimum fungicidal concentration (MFC) were applied to study the effects of some antifungal agents against *C albicans* Amphotericin B flucytosine and bifonazole produced total growth inhibition Clotrimazole itraconazole ketoconazole and miconazole produced partial growth inhibition The values of ICV and MFC were higher than those of IC 1/2 and IC 30

Gosch (1993) found out the minimum inhibitory concentrations (MIC) of tioconazole ranged from 0 03 to 4 µg/ml

The sensitivity of 28 dermatophyte strains and 25 yeast strains to various antifungal agents was investigated by Yucel (1993) MIC values of the various antifungals tested were diverse and there was significant increase in the MIC values after the addition of serum and keratin to the nutrient broth medium

The new *In vitro* method based on glucose consumption for determining antifungal activities against Aspergillus was developed by Garrigues *et al* (1994) Partial inhibition induced by low concentration of antifungal agent was quantifiable by his new method

Pawlik and Macura (1995) studied the susceptibility of 97 fungal strains to fluconazole by using the dilution method Minimum inhibitory concentration varied from

0 01100 mg/litre although growth of 75 2 per cent of the strains was considerably inhibited at 0 1 mg/litre

b) Plant extract \ essential oil

Frazier (1967) reported that essential oils of certain spices possess antimicrobial activities Oil of cinnamon was fairly effective against yeast and quite effective against bacteria At high concentration it permitted the mould mycelial growth but asexual spore formation was inhibited

The growth of *Aspergillus parasiticus* strain NRRL 2999 was affected by concentrations of cinnamon as low as per cent (200 µg /ml) Growth was reduced 13 31 per cent by concentrations of cinnamon ranging from 0 02 2 0 per cent (Bullerman, 1974)

Bullerman *et al* (1977) reported that cinnamon oil, clove oil cinnamic aldehyde and eugenol inhibited *Aspergillus parasiticus* growth and its subsequent toxin production in yeast sucrose broth medium. Cinnamon and clove are oils were more inhibitory at 125 ppm

Among the fifteen tested compounds by Yousef *et al* (1978) Cinnamaldehyde possessed the highest fungistatic and fungicidal activity towards *Microsporum audouni* and *T mentagrophytes*

Orthomethoxycinnamaldehyde (OMCA) inhibited the growth of *Aspergillus parasiticus A flavus A ochraceus and A versicolor* at 100 200 µg/ml and substantially inhibited mycotoxin (aflotoxin, ochratoxin, sterigmatocystin) production at 6 25 50 µg/ml It also had a strong inhibitory action against *Trichophyton mentagrophytes T rubrum Epidermophyton floccosum Microsporum gypseum M canis Cryptococcus neoformans* and *Candida albicans* at 3 12 50 µg/ml (Morozumi 1978)

In tests with 31 *Candida albicans* and 33 *Cryptococcus neoformans* strains with eugenol (a major constituent of oil of clove) the mean MIC were 625 and 293 µg/ml respectively and MFC were 1209 and 521 µg/ml respectively (Boonchird and Flegel 1982)

Fuzellier *et al* (1982) tested the antifungal activity of an aqueous extract of *Cassia alata* against *Trichophyton rubrum* (two isolates) and one isolate each of *Trichophyton mentagrophytes var interdigitale T mentagrophytes Microsporum gypseum M canis Epidermophyton floccosum Candida tropicalis C albicans* and *Cryptococcus neoformans* It showed antifungal activity comparable to that of aloeemodin, anthrone emodin and rhein (anthronicand anthroquinoic derivatives)

Essential oil from leaves of *Cinnamomum zeylanicum* was tested against several dermatophytes Candida spp and *Aspergillus fumigatus* by Saksena (1984) and it was found to possess considerable antifungal activity

Fourty essential oils were evaluated *In vitro* against 20 fungal isolates including 10 isolates of Rhizopus three of Mucor three of *Aspergillus flavus* and two of *A parasiticus* by Thompson and Cannon (1986) Seven of the oils found to be most effective included bay Cinnamon (bark and leaf) clove pimenta (berries and leaf) and thyme complete inhibitor of mycelial growth at 1000 ppm for Rhizopus spp and 500 ppm for Mucor and Aspergillus spp Clove oil was the most effective completely inhibiting all test fungi at 100 ppm The essential oil of leaves of *Cymbopogon martini* (lemon grass) exhibited fungi toxicity against three species of Aspergilli (namely *A flavus Aspergillus fumigatus and A parasiticus*) at 3000 2000 and 900 ppm, respectively (Misra *et al* 1988)

While screening the essential oils from leaves of 11 spp of higher plants for their fungitoxicity against *Aspergillus flavus* at 2000 3000 4000 and 5000 ppm, the oils of *Chenopodium ambrosioides Cinnamum zeylanicum Citrus medica Melaleuca leucadendron Ocimum canum* and *O gratissium* proved most effective inhibiting the test fungus at 2000 ppm The others were effective at higher concentration (Mishra *et al* 1989)

The essential oil of *Cymbopogon citratus* (lemon grass) was evaluated using fungistatic (MIC and agar diffusion tests) and fungicidal (spore germination studies) by Onawunmi (1989) Appreciable activity was observed against various isolates of Candida (*C albicans* and *C pseudotropicalis*) and clinical isolates of *Aspergillus fumigatus Microsporum gypseum* and *Trichophyton mentagrophytes*

Torremelis *et al* (1989) reported that decoction from *Cymbopogon citratus* (Lemon grass)leaves had an inhibitory effect on *Aspergillus nidulans*

Mangiarotti *et al* (1990) tested the essential oils of *Cinnamum zeylanicum* (bark and leaf) *Satureja hortensis Rosmarinus officinalis* and *Cymbopogon citratus* on 113 fungal strains representing 18 genera of yeasts and filamentous fungi including dermatophytes and moulds Dermatophytes were most sensitive to four essential oils particularly to cinnamon bark oil

Shadab qamar *et al* (1992) tested the essential oils from local and their cultivars against eight pathogenic fungi and *Saccharomyces cerevisiae* The greatest inhibitory activity was shown by a local oil which was stored for a peroid of two years This completely inhibited the growth of *Monilia sitophila* at 500 ppm, 1000 ppm for *Penicillium digitatum* and 1500 ppm for *Aspergillus fumigatus*

Antifungal activity of the essential oil of medicinal plants have been reported against different animal pathogens (Sudhadevi and Pillai, 1993)

Zhang (1995) studied the antifungal properties of cinnamaldehyde against 22 spp (31 strains) of opportunistic fungi It had antifungal activity against all fungi tested The MIC was 0 0625 1 0 mg/ml and MFC was 0 125 5 0 mg/ml

Materials and Methods

MATERIALS AND METHODS

Isolation of fungus from mastitis milk samples

Mastitis milk samples

For conducting the present study on the prevalence of yeast and yeast like fungal organism in bovine mastitis a total of 200 milk samples were collected from 161 cows clinically suffering from mastitis These cases were presented in any of the following institutions

1) Veterinary Hospital Kokkalai
2) Kerala Agricultural university Veterinary hospital Mannuthy
3) Veterinary Hospital/dispensaries located near Trichur

The history of the case nature of inflammation systemic reactions if any nature of the milk etc were noted before the collection of milk samples Mid stream milk was collected aseptically from each teat into sterile bottle and transported to the laboratory for further processing

Isolation of fungus

A sterile platinum loopful of milk from each sample was streaked separately on to one Tryptone soya agar (appendix 1H) and one Sabouraud s dextrose agar (appendix 1D) plate and the inoculated plates were incubated at 37°C for 24 48 hours for the isolation of bacteria, and yeast and yeast like fungal organisms Those plates with no discernable growth was further incubated for seven days before declaring it as negative for yeast or yeast like fungi

One loopful of milk from the above sample was inoculated on to Sabouraud s Dextrose Agar (SDA) separately by point inoculation at three (or) four sites and incubated at room temperature (25°C) for a period of two weeks for isolating filamentous fungal organism (mould)

After observing the growth the colony characters were examined and recorded Stock cultures were made on SDA slants from the pure cultures and stored at 4°C till the identification of organisms

Identification of yeast and yeast like fungi

For the identification of yeast and yeast like fungi subcultures were made on SDA in petriplates from the slant culture The identifications were done as per the guidelines recommended by and Davise Honig Larone (1976) and Yousef Al Doory (1980)

The isolates of yeast recovered from the milk samples were identified based on their colony characters microscopic morphology growth at 25°C with cycloheximide presence or absence of capsule growth on corn meal agar containing Tween 80 germ tube test urease test at 25°C growth at 37°C on SDA, growth in Sabouraud s dextrose broth sugar fermentation, containing dextrose maltose sucrose lactose and galactose and sugar assimilation test employing dextrose maltose sucrose lactose galactose melibiose cellobiose inositol xylose raffinose trehalose and dulcitol

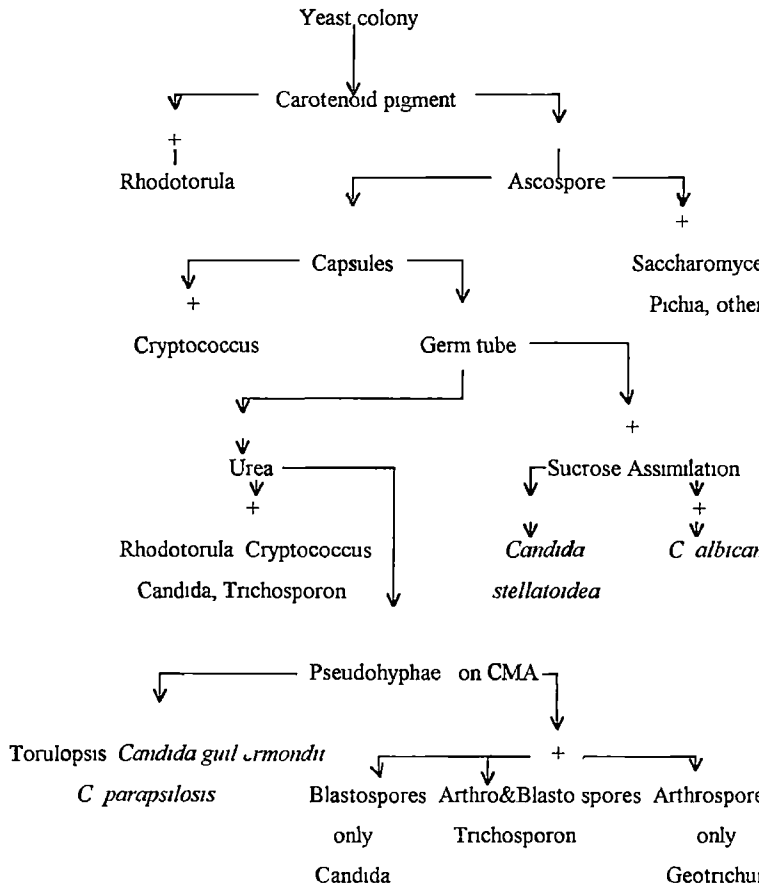
Wet mount

A drop of water was placed on a clean glass slide and a portion of the unknown yeast was removed with a sterile inoculating needle and light suspension was made It was covered with a coverslip and examined microscopically for ascospore formation

Ascospore formation was confirmed by use of an ascospore stain (appendix 1G) of an air dried smear

A flow chart used for the identification is given below

Identification of yeast and yeast like fungi



India ink mount

A drop of India ink was placed on a glass slide with a portion of unknown yeast colony Then it was covered with coverslip and examined in the microscope under high power for the presence of capsule

Corn meal agar cut streak culture

Plates of corn meal agar (appendix 1B) were inoculated using an inoculating needle by cutting through the agar The plates were incubated at 25°C for four days A part of the agar with growth, from the cut area was placed on a slide with a drop of lactophenol cotton blue A coverslip was placed over this and pressed down It was examined both under low power and high power to discern sporulation of the yeast if any

Germ tube test

The yeast colony was taken from the culture plate and emulsified in 0 5ml of sterile bovine serum The tubes were incubated for two and a half hours in a 36°C water bath A drop of the emulsion was transferred to a slide and examined under low power and high power magnification of a compound microscope

Urease test at 25°C

Christensen s urease agar (appendix 1H) slants were prepared and inoculated with the yeasts This was incubated at 25°C for a week The test was recorded as positive when the colour of the medium changed to red violet

Growth in Sabouraud s Broth

Saoouraud s dextrose broth (appendix 1E) was prepared and inoculated with the yeasts This was incubated at 37°C for 24 to 48 hours and examined for the type of growth namely narrow surface film with bubbles wide surface film upsides of tube pellicle forms and no surface growth

Sugar fermentation test

Three day old yeast culture was emulsified in sterile distilled water to get a density equal to tube one of Mcfarland s standard A suspension of 0 2 ml was added aseptically into the fermentation tubes (appendix 1C) containing the appropriate sugar with Durham s tube One tube was inoculated without sugars to serve as a control All the tubes were incubated at 37°C for seven days Change in colour consequent to the lowering of pH due to accumlation of acidic break down products of sugar fermentation was looked for Similarly gas collected in Durham s tube was also noted for

Sugar assimilation test

Three day old yeast culture was emulsified in sterile distilled water to get a density equal to tube one of Mcfarland s standard A suspension of 0 2 ml was added aseptically into each tube of medium (appendix 1A) containing the appropriate sugar for assimilation and a tube of yeast nitrogen base without any carbon source was used as a control All tubes were incubated at 37°C and cultures were examined over a period of 7 14 days for dense turbidity due to growth

Growth in SDA with cycloheximide at 25°C

Plates of SDA with cycloheximide (appendix 1F) were inoculated with a portion of unknown yeast colony and incubated at room temperature for five to seven days and the plates were examined for the presence of growth

Identification of mould

The filamentous mould isolate from milk sample was grown on SDA by point inoculation The plates were incubated at room temperature and observed daily for

a the rate of growth

b general topography

c texture

d surface pigmentation and

e reverse pigmentation

Microscopic examination was carried out using the adhesive tape technique

Adhesive tape technique

After obtaining sufficient growth and sporulation an adhesive transparent cello tape was touched over the growth This tape was transferred to a microscope slide on which a drop of lactophenol cotton blue (LPCB) was placed The microscopic characters of the filamentous fungi were examined first under low power and then under high power magnification

Slide culture

A bent glass rod was placed in a sterile petridish. Flamed clean glass microscope slide was placed on the glass rod One cm square agar block was cut with a sterile scalpel from the SDA plate and the block was transferred to the center of the glass slide The fungus was inoculated on to the center of four sides of the agar block with a heavy nichrome wire needle (22 gauge) A flamed coverslip was placed over the block and slight pressure was applied to ensure adherence, Eight ml of sterile water was poured to the bottom of the petridish and the dish cover was replaced and incubated at room temperature The block was examined periodically for growth and water was added if the plate begins to dry out The coverslip was removed carefully when the sporulation was well developed with a forceps and placed on a drop of LPCB on a second slide The agar block was gently flipped off the original slide with a forceps and discarded into a container of antifungal disinfectant A drop of LPCB was placed on the slide and new coverslip was placed over it and examined under microscope

Antifungl Susceptibility Test

Antifungal Chemotherapeutic agent

Filter paper disc agar diffusion method by Bauer *et al* (1966) was adopted in the present study

Preparation of disc

Paper discs of six mm diameter were cut from Whatman No 1 filter paper and dispensed in clean stoppered vials and were then sterilized in hot air oven at 140°C for 60 minutes

Preparation of Antifungal solution

The antifungal agents namely Griseofulvin (Tab Grisovin 200 mg) Itraconazole (Cap Canditral 100 mg) Ketoconazole (Tab keto zole 200mg) and P maricin (Natamycin 5% suspension) were procured from the local market

Griseofulvin tablet was crushed into powder and dissolved in acetone and diluted in 0 01 M PBS pH 7 0 Ketoconazole tablet was pulverized and dissolved in dimethylformamide and diluted in 0 01 M PBS at pH 7 Itraconazole powder also was dissolved in dimethylformamide and diluted in 0 01 M PBS pH 7 0 Natamycin suspension was diluted in 0 01 M PBS pH 7 0

The final concentration of these antifungal agents were adjusted as follows

Agents	Dilutions		
1 Griseofulvin	250µg	125µg	50µg
2 Itraconazole	0 03125µg	0 156µg	0 0078µg
3 Ketoconazole	100µg	80µg	60µg
4 Pimaricin	5µg	2 5µg	1 25µg

Preparation of antifungal sensitivity discs

The different diluted antifungal agents were adsorbed on to the sterilized discs using micropipette The quantity adsorbed per disc was 10 µl Discs were dried at room temperature and stored in tightly capped containers at 4°C Various concentrations of the drug of different antifungal agents were made

Readymade disc Readymade discs were purchased from Hi media for amphotericin B clotrimazole fluconazole and nystatin

1	Amphotericin B	100 units
2	Clotrimazole	10 µg
3	Fluconazole	10 µg
4	Nystatin	100 units

Antifungal activity of plant extracts

Extraction of Essential oils

Cinnamon leaf oil

Five hundred gram of cinnamon leaf was hydrodistilled for 45 minutes to four hours in a Clevenger apparatus and the oil was extracted

Lemon grass oil

Five hundred gram of lemon grass was hydrodistilled for 45 minutes in a Clevenger apparatus and the oil was extracted

Clove oil

Commercially available clove oil was purchased and used for the study

Cassia alata

Cassia alata leaf was dried in the shade for 7 days and then finely powdered and alkaloid was extracted by methanol and aqueous extraction

	Plant extracts	Concentration per disc
1	Cinnamon Leaf oil	1/10 1/20
2	Clove oil	1/10 1/20
3	Lemon grass oil	1/10 1/20
4	*Cassia alata*	1/10 1/20

Preparation of Plant essential oil / plant alkaloid sensitivity discs

Dilutions 1 10 and 1 20 of plant essential oils namely Cinnamon leaf oil clove oil and lemon grass oil were made in rectified spirit and 10 µl was placed on each pre sterilized filter paper disc

Methanol extract of *Cassia alata* was diluted in methanol and various dilutions of extract 1 10 and 1 20 were absorbed to disc and used

The fungal isolates from clinical cases of bovine mastitis were tested for their sensitivity for these agents by agar diffusion method Isolates were tested against amphotericin B clotrimazole fluconazole itraconazole ketoconazole nystatin pimaricin cinnamon leaf oil clove oil lemon grass oil and alkaloids of *Cassia alata*

Preparation of Inoculum

Yeast isolates

A suspension of cells from an overnight culture was made in yeast nitrogen broth The suspension was diluted to a final concentration of approximately 1×10^5 cells per ml

Filamentous fungi

Forty eight hour broth culture was homogenized by shaking and diluted to 1 100 (Holt and Newman 1972)

Inoculation

The yeast suspension and mould suspension thus obtained was adsorbed into a sterile cotton swab uniformly inoculated on yeast nitrogen base agar plates and allowed to dry for 10 minutes

Application of antifungal discs

The different antifungal discs were then placed on the medium suitably spaced with the help of sterile forceps The yeast inoculated plates were incubated at 37°C for 24 to 48 hours and the filamentous fungi inoculated plates were incubated at room temperature for five days

Reading and interpretation

At the end of the incubation period the plates were examined and the diameter of zones of complete inhibition was measured in mm with a scale

The zone of inhibition of each disc was measured in three different directions keeping the mid point of disc as the centre of the zone The mean of the measurement of inhibition was used for the interpretation of the results As adoptated by Shah *et al* (1986) the inhibitory zone of 15 mm and above was marked as highly sensitive 10 15 mm as moderately sensitive 5 10 mm as less sensitive and below 5 mm was declared as resistant

Results

RESULTS

In the present study milk samples collected were subjected to cultural screening to ascertain the association of pathogenic fungi and bacteria in the causation of mastitis A total of 200 samples from 161 cows were screened Out of the 200 samples 26 samples yielded fungal agents 121 samples were positive for bacteria and the remaining 53 were negative for fungal as well as bacterial agents The results are furnished in Table 1

Identification of the fungal isolates

Identity of the fungal isolates only was elucidated based on their colony characters microscopic morphology growth at 25°C with cycloheximide presence or absence of capsule growth on corn meal agar containing Tween 80 germ tube test urease test at 25°C growth at 37°C on SDA, growth in Sabouraud s dextrose broth, fermentation of sugars (dextrose maltose sucrose lactose and galactose) and sugar assimilation test employing dextrose maltose sucrose lactose melibiose cellobiose inositol xylose raffinose trehalose and dulcitol

1 Colony characters

In the present study five fungal isolates produced granular convex, discrete type of colonies four isolates produced convex, rough, spreading big colonies three isolates produced convex, rough discrete type of colonies two isolates produced smooth, creamy convex type of colonies One isolate produced flat dry circumscribed colony one isolate produced rough projection in the centre with papillomatous appearance One isolate produced dry dome shaped little waxy type of colony One isolate produced rough mucilaginous colony The colony character of the individual isolates are given in Table 2

2 Microscopic morphology

Microscopic study of the fungal isolates revealed oval budding yeast cell (eight isolates) oval elongated budding yeast cell with pseudohyphae (eight isolates) oval/ellip soidal yeast cell (two isolates) oval/elongated yeast cell (one isolate) and hyphae and arthrospores (one isolate) The microscopic morphology of the individual organisms are given in Table 3

3 Wet mount

Only one yeast and yeast like fungal isolate showed ascospore formation

4 Growth on SDA with cycloheximide at 25°C

In this test only five of the yeast and yeast like fungal isolates had shown the growth on SDA with cycloheximide The results are furnished in Table 4

5 Presence or absence of capsule

No fungal isolates revealed the capsule formation The results of the individual organism is furnished in Table 4

6 Growth on cornmeal agar containing Tween 80

In this study seven fungal isolates showed the blastospores anywhere along the pseudohyphae four fungal isolates produced true hyphae and arthrospores but no blastospores three isolates showed hyphae arthrospores and blastospores two solates carried blastospores along the pseudohyphae and giant mycelial cells one isolate showed a few very short pseudohyphae another solitary isolate showed fairly fine pseudohyphae and clusters of blastospores at septa yet another isolate showed no pseudohyphae and

the cells were small with terminal budding and one isolate produced only oval cells without pseudohyphae The results of the individual fungal isolates is shown in Table 4

7 Germ tube test

None of the isolates had shown the germ tube formation The results are shown in Table 4

8 Growth at 37°C on SDA

All the fungal isolates were able to grow on SDA at 37°C (Table 4)

9 Growth in Sabouraud's dextrose broth

Seven fungal isolates produced narrow surface film with bubbles at the rim Seven other isolates showed pellicle formation, while six other isolates showed no surface growth The results are furnished in Table 4

10 Urease test at 25°C

Four of the isolates were positive for urease test while 16 were negative for this test The results are furnished in Table 4

11 Sugar fermentation test

Twelve isolates produced both acid and gas three produced only acid and five failed to produce either acid or gas from dextrose fermentation

When tested for fermentation of maltose three isolates were found to produce both acid and gas six produced only acid and 11 isolates produced neither acid nor gas

Sucrose fermentation test revealed that three isolates could produce both acid and gas Eight isolates produced only acid while nine failed to produce acid as well as gas

No isolate was able to produce acid and/or gas in lactose fermentation test.Eight isolates produced both acid and gas three isolates produced only acid and nine isolates did not produce either acid or gas in galactose fermentation

The results of the fermentation test are furnished in Table 4

12 Sugar assimilation test

The number of isolates which could assimilate various sugars were dextrose and galactose all 20 isolates maltose and sucrose 16 lactose three melibiose two cellobiose seven xylose, 19 raffinose, seven, trehalose, 16 and dulcitol, only one None of the isolates could assimilate inositol The results of sugar assimilation test are furnished in Table 5

The characteristics exhibited by the isolates subjected to various tests were compared with the standard organisms Seven of the isolates were identified as *C tropicalis* (Plate 1 and 2) two as *C parapsilosis* (Plate 3 and 4) one as *C guillermondii* (Plate 5 and 6) four as *G candidum* (Plate 7 and 8) three as *T cutaneum* (Plate 9 and 10) one as *S cerevisiae* (Plate 11 and 12) one as Torulopsis spp (Plate 13 and 14) and one as *R. rubra* (Plate 15 and 16)

The major pathogen isolated was *C tropicalis* which constituted 27 percent The other organisms in the decreasing order were *G candidum* (15 38%) *T cutaneum* (11 53%) *C parapsilosis* (7 68%) *C guillermondii* (3 84%) *S cerevisiae* (3 84%) Torulopsis spp (3 84%) and *R. rubra* (3 84%)

Mould

The filamentous moulds isolated from milk samples were identified based on the rate of growth, general topography texture surface pigmentation, reverse pigmentation and microscopic examination

Each of the six mould isolates showed differences with respect to the various properties The details are furnished in Table 6

The filamentous mould isolates were Penicillium spp (7 69%) Sepedonium spp (3 84%) *Aspergillus ochraceus* group (3 84%) *Cladosporium carrionii* (3 84%) and *Trichophyton verrucosum* (3 84%)

Colony characters and microscopic morphology of the mould isolates are depicted in Plate 17 to 33

Organisms isolated versus changes in udder and milk

C tropicalis in 75 percent of the cases produced chronic mastitis in which mild fibrosis of udder reduction in milk yield and straw yellow coloured milk with flakes were noticed

Mastitis due to *G candidum* was chronic in nature with reduction in milk yield and the milk was watery with clots

Mastitis due to *T cutaneum* was of chronic nature with swollen udder and cream coloured milk

C parapsilosis produced chronic mastitis with flakes in the milk

Mastitis caused by *C guillermondii* was chronic type and mild fibrosis of udder was noticed

In *S cerevisiae* induced mastitis the milk was greyish with flakes

Torulopsis spp produced cream coloured watery milk with flakes Mastitis due to *R. rubra* was chronic type and flakes were noticed in the milk

In majority of the cases mould produced chronic mastitis characterised by hardness of udder with reduction in milk yield and straw yellow coloured milk viscid in consistency

The correlation between the symptoms of fungal mastitis and the associated fungal isolates are given in Table 7

Antifungal susceptibility test

In vitro antifungal chemotherapeutic sensitivity tests were carried out on all the 20 yeast and yeast like fungal isolates and the details are shown in Table 8 and figure 1 and 2

Among the seven *C tropicalis* tested for *in vitro* antifungal sensitivity six were highly sensitive to Clotrimazole (10µg) two to Fluconazole (10µg) four to Nystatin (100u) five to Itraconazole (0 015µg) two to Itraconazole (0 0078µg) two to Ketoconazole (80µg) and one to Pimaricin (2 5µg)

Moderate sensitivity of three isolates to Amphotericin B (100u) one to Clotrimazole (10µg) one to Fluconazole (10µg) three to Nystatin (100u) two to Itraconazole (0 015µg) four to Itraconazole (0 0078µg) five to Ketoconazole (80µg) three to Ketoconazole (60µg) four to Pimaricin (2 5µg) and one to Pimaricin (1 25µg)

Less sensitivity of four isolates to Amphotericin B (100u) two to Itraconazole (0 0078µg) four to Ketoconazole (60µg) two to Pimaricin (2 5µg) and three to Pimaricin (1 25 µg)

Four isolates were resistant to Fluconazole (10µg) and three to Pimaricin (1 25µg)

C parapsilosis were found to be highly sensitive to Clotrimazole (10µg) Nystatin (100u) Itraconazole (0 031 µg) Itraconazole (0 015µg) Ketoconazole (100µg) Pimaricin (5µg) and moderately sensitive to Amphotericin B (100u) Itraconazole (0 0078 µg) Ketoconazole (80µg) and Pimaricin (2 5µg) One isolate was moderately sensitive to Ketoconazole (60µg) and Pimaricin (1 25µg) while the other was less sensitive

to Ketoconazole (60µg) and Pimaricin (1 25 µg) Both isolates were resistant to Fluconazole (10µg)

C guillermondii isolate was highly sensitive to Clotrimazole (10µg) Itraconazole (0 031 µg) Itraconazole (0 0156µg) Ketoconazole (100µg) and Pimaricin (5µg) Moderately sensitive to Amphotericin B (100u) Nystatin (100u) Itraconazole (0 0078µg) Ketoconazole (80µg) and Pimaricin (2 5µg) least sensitive to Ketoconazole (60µg) and resistant to Pimaricin (1 25µg) and Fluconazole (10µg)

All the four isolates of Geotrichum candidum were found to be highly sensitive to Clotrimazole (10µg) Nystatin (100u) Itraconazole (0 031 µg) Itraconazole (0 015µg) Itraconazole (0 078µg) Ketoconazole (100µg) Ketoconazole (80µg) and Ketoconazole (60 µg) moderately sensitive to Amphotericin B (100u) and resistant to Fluconazole (10µg) and Pimaricin (5µg)

Among the three *Trichosporon cutaneum* isolates tested all were highly sensitive to Clotrimazole (10µg) Ketoconazole (100µg) Ketoconazole (80µg) and ketoconazole (60µg) One isolate was highly sensitive to Fluconazole (10µg) Nystatin (100u) Itraconazole (0 031µg) and Itraconazole (0 015µg) All the three isolates were moderatley sensitive to Amphotericin B (100u) two to Pimaricm (5µg) Nystatin (100u) and one to Itraconazole (0 0078µg) Two isolates were least sensitive to Fluconazole (10µg) and one to Itraconazole (0 031µg) Pimaricm (5µg) Pimaricin (2 5µg) and Nystatin (100u) All the three isolates were resistant to Pimaricin (1 25µg) Two isolates were resistant to Itraconazole (0 015µg) Itraconazole (0 0078µg) Pimaricin (2 5µg) and one was resistant to Itraconazole (0 031 µg)

Saccharomyces cerevisiae was highly sensitive to Amphotericin B (100u) Clotrimazole (10µg) Nystatin (100u) Itraconazole (0 031µg) Itraconazole (0 015µg) Itraconazole (0 0078µg) and Pimaricin (5 µg) moderately sensitive to Ketoconazole

(100µg) Pimaricin (2 5 µg) less sensitive to Ketoconazole (80µg) and resistant to Ketoconazole (60 µg) Pimaricin (1 25µg) and Fluconazole (10µg)

Torulopsis spp was highly sensitive to Clotrimazole (10µg) Nystatin (100u) Itraconazole (0 031µg) Itraconazole (0 015µg) Itraconazole (0 0078µg) Ketoconazole (100µg) and Ketoconazole (80 µg) Moderately sensitive to Amphotericin B (100u) Ketoconazole (60 µg) Pimaricin (5µg) and resistant to Fluconazole (10µg) Pimaricin (2 5µg) and Pimaricin (1 25 µg)

Rhodotorula rubra was highly sensitive to Itraconazole (0 031µg) Ketoconazole (100µg) Ketoconazole (80µg) Pimaricin (5µg) Pimaricin (2 5µg) and Pimaricin (1 25µg) moderately sensitive to Clotrimazole (10µg) Nystatin (100u) Itraconazole (0 015µg) Itraconazole (0 0078µg) Ketoconazole (60 µg) less sensitive to Amphotericin B (100µg) and resistant to Fluconazole (10µg)

All the 20 yeast and yeast like fungal isolates tested for *in vitro* sensitivity against Griseofulvin (250 µg) showed resistance

Mould

In vitro antifungal chemotherapeutic sensitivity tests were carried out on all the six mould isolates and the details are given in Table 10

T verrucosum was moderately sensitive to Pimaricin (5µg) Itraconazole (0 031µg) and Clotrimazole (10µg) less sensitive to Pimaricin (2 5µg) Itraconazole (0 015µg) and resistant to Amphotericin B (100u) Ketoconazole (100µg) and Nystatin (100u)

The solitary isolate belonging to *A ochraceous* group was found to be moderately sensitive to Clotrimazole (10µg) less sensitive to Pimaricin (5µg) and Amphotericin B (100u) and resistant to Itraconazole (0 031µg) Ketoconazole (100µg) and Nystatin (100u)

C carrionii was moderately sensitive to Ketoconazole (100µg) and Itraconazole (0 031µg) less sensitive to Ketoconazole (60µg) Pimaricin (5µg) Itraconazole (0 015µg) Amphotericin B (100u) and Nystatin (100u) and resistant to Clotrimazole (10µg)

Sepedomum spp was moderately sensitive to Clotrimazole (10µg) Itraconazole (0 031µg) and Pimaricin (5µg) less sensitive to Amphotericin B (100u) Itraconazole (0 015µg) Ketoconazole (100µg) and Pimaricin (2 5µg) and resistant to Itraconazole (0 0078µg) Ketoconazole (60µg) Ketoconazole (80µg) Nystatin (100u) and Pimaricin (1 25µg)

One Penicillium spp was found to be moderately sensitive to Clotrimazole (10 g) less sensitive to Itraconazole (0 031µg) and resistant to Amphotericin B (100u) Ketoconazole (100µg) and Pimaricin (5µg) The other Pemcillium isolate was highly sensitive to Itraconazole (0 031µg) moderately sensitive to Itraconazole (0 015 ơ) Pimaricin (5µg) and Clotrimazole (10µg) less sensitive to Amphotericin B (100u) Nystatin (100u) Ketoconazole (100µg) and Pimaricin (2 5µg) and resistant to Ketoconazole (60µg) Ketoconazole (80µg) and Pimaricin (1 25µg)

All the six mould isolates tested for *in vitro* sensitivity against Fluconazole (10 g) and Griseofulvin (250µg) showed resistance

In vitro antifungal susceptibility of mould isolate showed that 16 6 per cent o he isolates were highly sensitive to Itraconazole (0 031µg) 83 per cent moderately

sensitive to Clotrimazole (10µg) 50 per cent to Pimaricm (5µg) and Itraconazole (0 031µg) 16 6 per cent to Ketoconazole (100µg) 83 per cent less sensitive to Amphotericin B (100u) and Clotrimazole (10µg) 33 per cent to Ketoconazole (100µg) and Pimaricin (5µg) 16 6 per cent to Itraconazole (0 031µg) and cent per cent resistance to Fluconazole (10µg) and Griseofulvin (250µg)

Antifungal activity of Plant Extract

The antimicrobial activity of essential oil extracted from Cinnamon leaf (CLO) Clove (CO) Lemon grass (LGO) aqueous and methanol extracts from Cassia alata were tested *in vitro* against the yeast and yeast like fungal isolates and the observations are presented in Table 9 and Fig 3

Aqueous and methanol extract of *Cassia alata* tested failed to exhibit antifungal activity against any of the fungal isolates

It was found that the essential oil of Cinnamon leaf clove and lemon grass showed varying degrees of antifungal properties

Out of three essential oils tested Cinnamon leaf oil was found to be active against all the yeast and yeast like fungal isolates tested at 1 in 10 dilution The highest zone of inhibition was for *Geotrichum candidum* (26 mm)

Five per cent of the isolates showed high sensitivity to CLO at 1 in 10 dilution while 25 per cent were moderate sensitive and 10 per cent least sensitive

Twenty five per cent of the isolates showed high sensitivity to CLO at 1 in 20 dilution while 30 per cent were moderately sensitive 35 per cent less sensitive and 10 per cent resistant

Essential oil obtained from clove leaf showed high inhibitory action against 30 per cent moderate action against 30 per cent and low action against 40 per cent of the

yeast and yeast like fungal isolates at 1 in 10 dilution The highest zone of inhibition (20mm) was for *Rhodotorula rubra*

At 1 in 20 dilution, CO showed moderate activity against 30 per cent low activity against 45 per cent Twenty five per cent isolates were resistant

Essential oil obtained from lemon grass (1 10 dilution) showed the percentage of high, moderate and low activity against yeast and yeast like fungal isolates as 25 45 and 30 respectively The highest zone of inhibition (21mm) was for *Trichosporon cutaneum*

At 1 in 20 dilution it showed low activity against 40 per cent and resistance to 60 per cent yeast and yeast like fungal isolates

Among the seven *C tropicalis* tested for *in vitro* antifungal sensitivity four isolates were highly sensitive to CLO (1in 10) four isolates were moderately sensitive to CO (1in 10) and LGO (1 in 10) two isolates were moderately sensitive to CLO (1 in 10 and 1 in 20) Five isolates were less sensitive to CLO (1 in 20) and CO (1 in 20) Three isolates were moderately sensitive to CO (1 in 10) and LGO (1 in 10) Five isolates were resistant to LGO (1 in 20) and two isolates were resistant to CO (1 m 20)

One *C parapsilosis* isolate was found to be highly sensitive to CLO (1 in 10) and moderately sensitive to CLO (1 in 20) and CO (1 in 10) and less sensitive to CO (1 in 20) and LGO (1 in 10) The other isolate was moderately sensitive to CLO (1 in 10) LGO (1 in 10) and less sensitive to CLO (1 in 20) CO (1 in 10) and CO (1 in 20) Both the isolates were resistant to LGO (1 in 20)

C guillermondii was highly sensitive to CLO (1 in 10) moderately sensitive to CLO (1 in 20) CO (1 in 10) and LGO (1 in 10) less sensitive to CO (1 in 20) and resistant to LGO (1 in 20)

Four *Geotrichum candidum* isolates were found to be highly sensitive to CLO (1in 10) CLO (1 in 20) CO (1in 10) and LGO (1 in 10) moderately sensitive to CO (1 in 20) and less sensitive to LGO (1 in 20)

Among the three *Trichophyton cutaneum* isolates tested one isolate was highly sensitive to CLO (1 in 10) CLO (1 in 20) CO (1 in 10) and LGO (1 in 10) one isolate was moderately sensitive to CLO (1 in 10) and CO (1 in 20) two isolates were less sensitive to CO (1 in 10) and LGO (1 in 10) One isolate was less sensitive to CLO (1 in 10) and LGO (1in 20) and two isolates were resistant to CLO (1 in 20) and LGO (1 in 20)

Saccharomyces cerevisiae was highly sensitive to CLO (1 in 10) moderately sensitive to (1 in 20) and LGO (1 in 10) less sensitive to CO (1 in 10 and 1 in 20) and resistant to LGO (1 in 20)

Torulopsis spp was highly sensitive to CLO (1 in 10),moderately sensitive to CLO (I in 20) and LGO (1 in 10) less sensitive to CO (1 in 10) and resistant to CO (1 in 20) and LGO (1 in 20)

Rhodotorula rubra was highly sensitive to CO (1 in 10) moderately sensitive to CLO (1 in 10) CO (1 in 20) LGO (1 in 10) and less sensitive to CLO (1 in 20) and LGO (i in 20)

Mould

T verrucosum was found to be resistant to all the three essential oils tested

A ochraceous group was less sensitive to CLO at 1 10 dilution resistant to CLO at 1 20 and CO and LGO at 1 10 and 1 20 dilutions

C carrionii was moderately sensitive to CO at 1 10 less sensitive to CLO at I 10 CO at 1 20 and resistant to CLO at 1 20 LGO at 1 10 and 1 20 dilutions

Sepedonium spp was moderately sensitive to CLO at 1 10 CO at 1 10 ess sensitive to CLO at 1 20 CO at 1 20 and resistant to LGO at 1 10 and 1 20 dilutions

Penicillium spp were moderately sensitive to CLO at 1 10 dilution less sensitive at 1 20 dilution and resistant to CO and LGO at 1 10 and 1 20 dilutions

Table 1 Showing details regarding the number of cows and samples screened and case positive for bacteria and fungi

No of Animals	No of samples screened	No of samples +ve for			No of samples ve
		Bacteria	Fungus	Both	
161	200	121	17	9	53
		(60 5%)	(8 5%)	(4 5%)	(26 5%)

Table 2 Details of colony characters of yeast and yeast like fungal isolates

Isolate No	Colony Characters
1	Small granular type of discrete colonies
2	Rough mucilaginous in side
3A	Convex rough discrete colonies
3B	Granular convex creamy type of colonies
4	Convex rough discrete colonies
5A	Con ex spreading rough b g colonies
5B	Convex spreading rough big colonies
5C	Convex spreading rough big colonies
5D	Convex spreading rough big colonies
6	Granular con ex creamy very discrete piliform type of colonies
7	Granular yellowish rough, convex type of colonies
8	Rough eructing projection of the centre with papillamatous appearence
9	Flat dry circumcribed colonies
10	Dry dome shaped little waxy type of colonies
11	Granular convex creamy very discrete piliform type of colonies
12A	Granular dry sl ghtly yello v pigmented type of colonies
12B	Piliform type of colony fine and granular slightly p gmented (pink)
13	Smooth creamy con ex type of colonies
14	Smooth creamy convex type of colonies
15	Con ex rough discrete big colon es

Table 3 Microscopic morphology of yeast and yeast like fungal isolates

Isolate No	Microscopic morphology
1	Oval and elongated yeast cell
2	Oval elongated budding yeast cell and pseudohyphae present
3A	Round and oval budding yeast cell
3B	Oval budding yeast cell
4	Elongated budding yeast cell and pseudohyphae present
5A	Oval budding yeast cell
5B	Oval budding yeast cell
5C	Oval budding yeast cell
5D	Oval budding yeast cell
6	Oval budding yeast cell
7	Elongated yeast cell and pseudohyphae present
8	Oval/ellopsoidal yeast cell
9	Hyphae and arthrospores present
10	Oval/ellopsoidal yeast cell
11	Elongated yeast cell and pseudohyphae present
12A	Oval yeast cell and pseudohyphae present
12B	Small oval budding yeast cell
13	Oval elongated yeast cell and pseudhypl ae present
14	Round oval budding yeast cell and pseudohyphae present
15	Oval elongated yeast cell and pseudohyphae present

Table 4 Showing the characteristics of the yeast and yeast-like fungal isolates

Isolate No	Microscopic morphology on corn meal agar	Fermentation					Growth in Sabouraud's broth	Urease 25°C	Capsule	Growth at 25°C with cycloheximide	Growth at 37°C on SDA	Germ tube	Identity of isolate
		D	M	S	L	G							
1	Blastospores anywhere along pseudohyphae	AG	A	A	O	AG	narrow surface film with bubbles	O	O	O	+	O	*C tropicalis*
2	Few very short pseudohyphae	AG	AG	AG	O	AG	no surface growth	O	O	O	+	O	*Saccharomyces cerevisiae*
3A	Blastospores anywhere along pseudohyphae	AG	AG	AG	O	AG	narrow surface film with bubbles	O	0	+	+	O	*C tropicalis*
3B	Fairly short fine pseudohyphae clusters of blastospores at septa	AG	O	A	O	A	no surface growth	O	O	+	+	O	*C guilliermondii*
4	Hyphae arthrospore blastospore	A	A	O	O	O	pellicle forms	+	O	O	+	O	*Trichosporon cutaneum*
5A	True hyphae arthrospores no blastospores	O	O	O	O	O	pellicle forms	O	O	O	+	O	*Geotrichum candidum*
5B	True hyphae arthrospores no blastospores	O	O	O	O	O	pellicle forms	O	O	O	+	O	*Geotrichum candidum*
5C	True hyphae arthrospores no blastospores	O	O	O	O	O	pellicle forms	O	O	O	+	O	*Geotrichum candidum*
5D	True hyphae arthrospores no blastospores	O	O	O	O	O	pellicle forms	O	O	O	+	O	*Geotrichum candidum*

6	No psuedohyphae cells small term nal budding	AG O O O O	no surface growth	O	O	O	+	O	Torulopsis spp
7	Blastospores any where along pseudohyphae	AG A A O AG	narrow surface film	O	O	O	+	O	*C tropicalis*
8	Blastospores any where along pseudohyphae	AG A A O AG	narro surface film	O	O	O	+	O	*C trop cal s*
9	Hyphae arthrospore blastospores	A O O O O	pellicle forms	+	0	+	+	0	*T cutaneun*
10	Blastospores anywhere along pseudol yphae	AG AG AG O AG	narrow surface film with bubbles	O	O	+	+	O	*C trop cal s*
11	Blastospores anywl ere along pseudohyphae	AG A A O AG	narro surface film vith bubbles	O	O	0	+	O	*C trop cal s*
12A	Blastospores along pseuodol yphae giant mycelial cells	AG O A O A	no surface growth	O	O	O	+	O	*C parapsilosis*
12B	No pseudol vphae	O O O O O	no surface growth	+	O	O	+	O	*Rhodotorula rubra*
13	Blastospores along pseudohyphae giant mycelial cell	AG O A O A	no surface growth	O	O	O	+	O	*C parapsilos s*
14	Blastospores any vhere along pseudohyphae	AG A A O AG	narro surface film vith bubbles	O	O	O	+	O	*C trop calis*
15	H pl ae artl rospore blastospore	A O O O O	pellicle forms		O	+	+	O	*T ct taneun*

O negative A acid produced G gas produced + positive

D Dextrose M Maltose S Sucrose L Lactose G Galactose

Table 5 Results of carbohydrate assimilation Tests for yeast and yeast like fungal isolates

Isolate No	Dext rose	Malt ose	Sucr ose	Lact ose	Gala ctose	Meli biose	Cellob iose	Inos itol	Xyl ose	Raff inose	Treh alose	Dul citol	Organi m
1	+	+	+	o	+	o	o	o	+	o	+	o	*Cand da trop ca s*
2	+	+	+	o	+	o	o	o	o	+	+	o	*Sa cha omvces ce evi ae*
3A	+	+	+	o	+	o	+	o	+	o	+	o	*C trop ca*
3B	+	+	+	o	+	+	+	o	+	+	+	+	*C gu llermond*
4	+	+	+	+	+	o	+	o	+	+	+	o	*Tricho po on cu aneum*
5A	+	o	o	o	+	o	o	o	+	o	o	o	*Geotr chum cand dum*
5B	+	o	o	o	+	o	o	o	+	o	o	o	*G and d n*
5C	+	o	o	o	+	o	o	o	+	o	o	o	*G cand du n*
5D	+	o	o	o	+	o	o	o	+	o	o	o	*G cand d n*
6	+	+	+	o	+	o	o	o	+	+	+	o	Toru op s pp
7	+	+	+	o	+	o	o	o	+	o	+	o	*C trop ca s*
8	+	+	+	o	+	o	+	o	+	o	+	o	*C trop ca*
9	+	+	+	+	+	+	+	o	+	+	+	o	*Tricho po on cu ane n*
10	+	+	+	o	+	o	+	o	+	o	+	o	*C op a s*
11	+	+	+	o	+	o	o	o	+	o	+	o	*C op ca*
12A	+	+	+	o	+	o	o	o	+	o	+	o	*C pa ap o*
12B	+	+	+	o	+	o	o	o	+	+	+	o	*Rhodo oru a ub a*
13	+	+	+	o	+	o	o	o	+	o	+	o	*C pa ap o*
14	+	+	+	o	+	o	o	o	+	o	+	o	*C trop ca*
15	+	+	+	+	+	o	+	o	+	+	+	o	*Tc ane n*

Tables 6 Colony characters and microscopic morphology of mould isolates

Isolate No	Rate of growth	Colony morphology at 25°C	Microscopic morphology	organism
1	Moderately rapid mature within 7 days	Colonies are at first white and waxy then become fluffy and with age turn yellow Reverse is white	Septate hyphae bearing simple conidiophores The conidia may be simple oval thick walled and smooth	Sepedonium spp
2	Rapid mature within 4 days	Surface at first white then becoming very powdery bluish green with a white border Reverse is light	Septate hyphae with branched or unbranched conidiophores that have secondary branches known as metulae On the metulae arranged in whorls are flask shaped sterigmata that bear unbranched chains of round conidia. The entire structure forms the characteristic penicius' or brush appearance	Penicillium spp
3	Slow mature within 21 days	Dark surface flat with slightly raised center covered with velvetty grey green short napped mycelium Reverse is black	Hyphae septate with lateral and terminal conidiophores of varying size.Sporulation is of the Cladosporium type ie conidiophores produce long chains of smooth walled oval some what pointed conidia	*Cladosporium carr oni*
4	Moderately rapid mature with in 7 days	Surface has spreading white cottony areial mycelium later turning grey Reverse is grey	Septate hyphae with simple long conidiophores bearing vesicles mostly globose occasionally somewhat elongate Sterigmata in two series Conidial heads globose ocharaceous Shades Conidia globose smooth walled. Sclerotia not produced Conidia 1 8 2 7µ sterigmata(length) 9 14 4µ vesicle size(diameter) 11 7 to 18µ	*Asperg llus ochraceous* group

5	Slow mature in 7 10 days	Small heaped button like Texture slightly downy White Reverse brown	Hyphae with many chlamydospore (in chains) and some antler branches are seen	*Trichophyton verrucosum*
6	Rapid mature within 4 days	Surface at first white then becoming very powdery bluish green with a white border Reverse is light.	Septate hyphae with branched or unbranched conidiophores that have secondary branches known as metulae On the metulae arranged in whorls are flask shaped sterigmata that bear unbranched chains of round conidia The entire structure forms the characterstics Penicius or brush appearance	Penicillium spp

Table 7 Details regarding the changes in udder and milk samples and the type of fungal agent associated

Characteristics of milk samples	Organism isolated
Greyish watery milk gelatinous mass	Sepedonium spp
Cream colour with flakes seen	*Candida tropicalis*
Yellow in colour with flakes seen	Penicillium spp
Greyish in colour with flakes seen udder swollen treated with antibiotics	*Cladosporium carrionii*
Chronic mastitis mild fibrosis of udder Cow was treated with gentamicin and oxyteracycline It did not respond to the treatment	*Candida guillermondii*
Milk cream colour with flakes udder swelling noticed	*Trichosporon cutaneum*
Watery milk whitish flakes seen	*Geotrichum candidum*
Cream colour watery milk with whitish flakes seen	Torulopsis spp
Milk yellow in colur with flakes seen	*Aspergillus ochraceous* group
Milk blood tinged fibrosis of teat intermittent bleeding noticed	*Trichophyton verrucosum*
RH straw yellow colour watery consistency	*C tropicalis*
LH straw yellow colour watery consistency	Penicillium spp
Cream colour watery milk	*C tropicalis*
Cream colour udder swollen The cow was treated with gentamicin choramphenicol	*Trichosporon cutaneum*
Induration of udder milk straw coloured	*C tropicalis*
Chronic mastitis yellow milk with flakes	*C tropicalis*
Chronic mastitis flakes present in the milk	*C parapsilosis*
Chronic mastitis flakes present in the milk	*Rhodotorula rubra*
Thin milk with flakes	*C parapsilosis*
Cream colour with flakes seen	*C tropicalis*
Chronic mild induration of udder milk straw coloured with flakes	*C tropicalis*
Cream colured milk watery	*Trichosporon cutaneum*

Table 8 Sensitivity pattern of the yeast and yeast like fungal isolate against the common anti fungal agents
(Diameter of the zone of inhibition is given in mm)

Iso ate No	Organism	A (00u)	C (0µ g)	F (10 g)	G 2ᵓ0µg)	ᵓ (00u	I			K			P		
							003125 g)	(0 0 56µg)	0 0078 g)	(100 g	80µg)	60 ₂)	(5 g)	(25 g	(1 5 g)
1	*C trop cal s*	9	20	12	0	14	20	17	14	16	12	9	17	13	8
2	*Saccharon yces cerevis ae*	16	17	0	0	28	24	21	16	12	6	0	16	14	0
3A	*C trop cal s*	12	11	15	0	10	20	17	13	19	1ᵓ	12	18	14	0
3B	*C gu llern o id*	10	22	0	0	10	22	18	13	18	13	8	15	12	6
4	*Tr chosporon ci taneun*	12	24	30	0	ᵓ0	21	16	11	41	ᵓ2	21	12	7	0
ᵓA	*Geotr cu n cand dum*	10	19	0	0	26	22	18	15	28	22	18	0	0	0
5B	*Geotr cum cand dum*	10	19	0	0	26	22	18	1ᵓ	28	22	18	0	0	0
5C	*Geotr cu i cand du i*	10	19	0	0	26	22	18	15	28	22	18	0	0	0
ᵓD	*Geotr cu i ca id d i*	10	19	0	0	26	22	18	15	28	22	18	0	0	0
6	Torulopsis spp	10	26	0	0	20	40	22	15	20	1ᵓ	10	10	0	0
7	*C trop cal s*	10	20	0	0	28	26	20	15	16	13	10	15	8	0
8	*C trop cal s*	14	16	0	0	24	24	19	16	15	12	9	16	11	8
9	*Tr chosporon cutane m*	10	20	8	0	9	7	0	0	21	18	15	9	0	0
10	*C trop cal s*	12	17	20	0	12	15	11	8	19	16	14	19	15	10
11	*C trop cal s*	9	21	0	0	22	20	16	12	20	11	9	15	9	0
12A	*C paraps los s*	10	19	0	0	20	38	24	13	16	12	9	16	10	7
12B	*Rhodotorula rubra*	9	12	0	0	1ᵓ	18	14	11	22	18	12	24	20	15
13	*C paraps los s*	11	18	0	0	20	34	20	10	18	14	12	17	14	10
14	*C trop cal s*	9	16	0	0	16	18	13	10	20	12	9	1ᵓ	11	6
15	*T cutane n*	12	119	8	0	10	0	0	0	22	18	15	11	0	0

A Amphoterıcın B C Clotrımazole F Fluconazole G Grıseofulvın N Nystatın I Itraconazole K ketoconazole P Pımarıcın

Table 9 Sensitivity pattern of the yeast and yeast like fungal isolates against the plant extract/essential oil

(Diameter of the zone of complete inhibition is given in mm)

Isolate No	Organism	Cass a al ita		Cinnamon Leaf o l		Clo e oil		Lemon grass o	
		1/10	1/20	1/10	1/20	1/10	1/20	1/10	1/?0
1	*Cand da trop cal s*	0	0	9	6	12	7	12	6
2	*Saccharomyces cerev s ae*	0	0	17	10	9	6	10	0
3A	*C trop cal s*	0	0	18	8	7	0	7	0
3B	*C g llermond*	0	0	1ɔ	10	10	6	10	0
4	*Trichosporon cutane m*	0	0	20	15	18	13	21	8
ɔA	*Geotr cl ı ı cand dum*	0	0	26	20	1ɔ	10	15	6
5B		0	0	26	20	1ɔ	10	1ɔ	6
ɔC		0	0	26	20	15	10	15	6
5D		0	0	26	20	1ɔ	10	1ɔ	6
6	Torulopsis spp	0	0	18	10	9	0	10	0
7	*C tropicalis*	0	0	18	10	9	6	10	0
8	*C trop cal s*	0	0	20	12	10	6	12	6
9	*Tr cl osporon cı tane*	0	0	10	0	7	0	6	0
10	*C trop cal s*	0	0	1?	8	10	7	7	0
11	*C rop cal s*	0	0	17	9	10	6	10	0
12A	*C paraps los s*	0	0	16	11	10	6	9	0
12B	*Rl odotorı la rubra*	0	0	11	8	20	10	14	6
13	*C paraps los s*	0	0	11	8	9	7	10	0
14	*C trop cal s*	0	0	10	8	8	0	8	0
15	*T c tane*	0	0	9	0	8	0	7	0

Table 10 Sensitivity pattern of the mould isolates against the common antifungal agents

(Diameter of the zone of complete inhibition is given in mm)

Isola e No	Organism	A(100u)	C(10µg)	F(10µg)	G(250µg)	N(100u)	I			K			P		
							0 0312 µg	0156µg	0 00078µg	100µg	80µg	60µg	5µg	2 5µg	1 25 µg
1	Sepedonium spp	9	12	0	0	0	11	7	0	0	0	0	11	7	0
2	pen c ll um spp	7	10	0	0	8	17	14	8	8	0	0	14	9	0
3	*Cladospon n carrion*	8	0	0	0	8	12	8	0	14	10	7	8	0	0
4	*Asperg llus ochraceo s* group	7	10	0	0	0	0	0	0	0	0	0	8	0	0
5	*Trcl opl y o verri cos n*	9	10	0	0	0	10	7	0	0	0	0	10	7	0
6	Penic ll um spp	0	10	0	0	0	8	0	0	0	0	0	0	0	0

A Ampl otericin B C Clotrimazole F Fuconazole G Griscoful in N N statin I Itraconazole K Ke oconazole P Pimaricin

Table 11 Sensitivity pattern of the mould isolates against the plant extract/essential oils
(Diameter of the zone of complete inhibition is given in mm)

Isolate No	Organism	*Cassia alata*		Cinnamon leaf oil		Clove oil		Lemon grass oil	
		1/10	1/20	1/10	1/20	1/10	1/20	1/10	1/20
1	Sepedonium spp	0	0	10	6	11	8	0	0
2	Penicillium spp	0	0	10	6	0	0	0	0
3	*Cladosporium carrionii*	0	0	7	0	10	6	0	0
4	*Aspergillus ochraceous* group	0	0	8	0	0	0	0	0
5	*Trichophyton verrucosm*	0	0	0	0	0	0	0	0
6	Penicillium spp	0	0	10	7	0	0	0	0

Fig 1 SENSITIVITY PATTERN OF YEAST AND YEAST LIKE FUNGAL ISOLATES AGAINST COMMON ANTIFUNGAL AGENTS

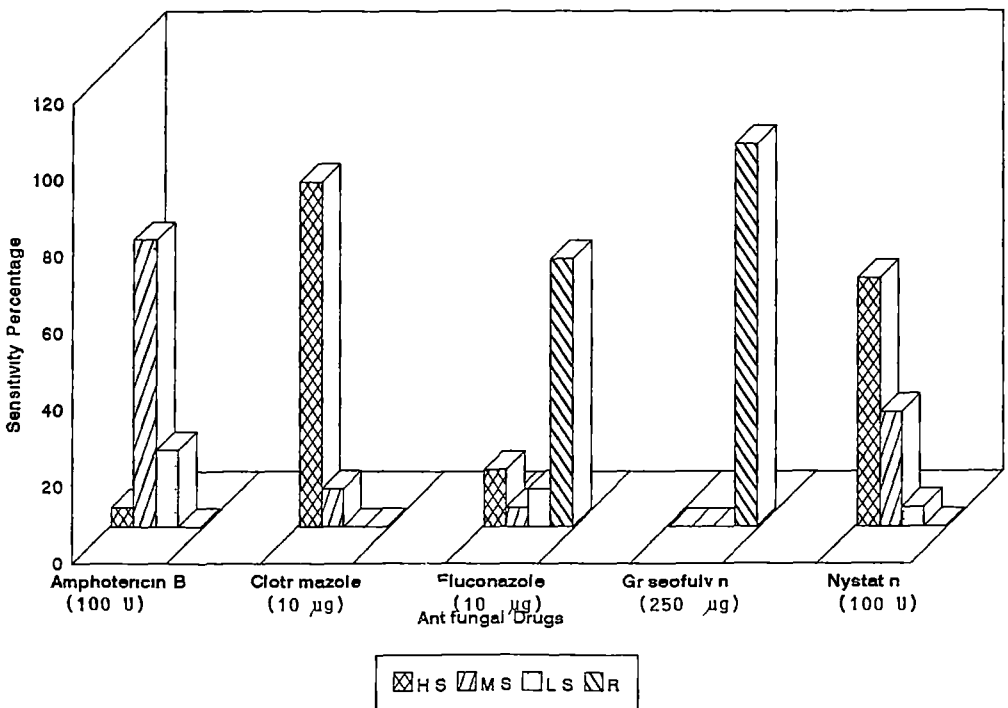
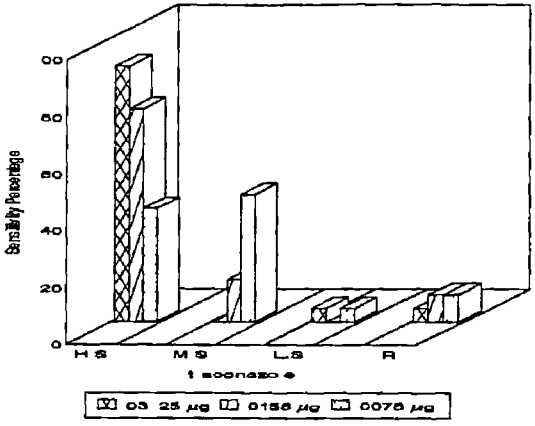
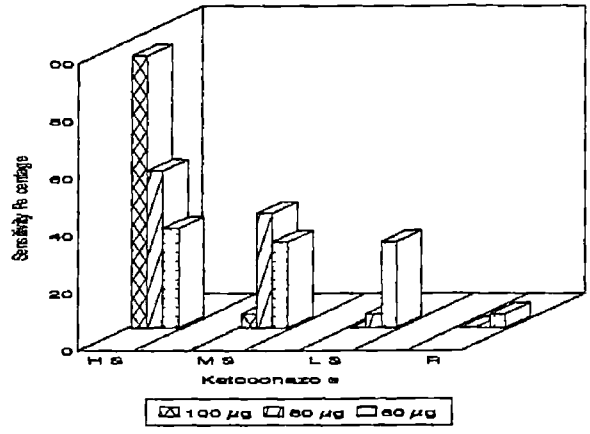


Fig 2 SENSITIVITY PATTERN OF YEAST AND YEAST LIKE FUNGAL ISOLATES AGAINST COMMON ANTIFUNGAL AGENTS AT VARYING CONCENTRATIONS





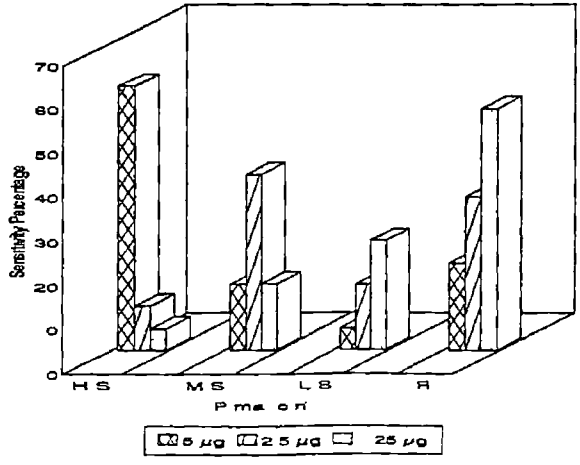
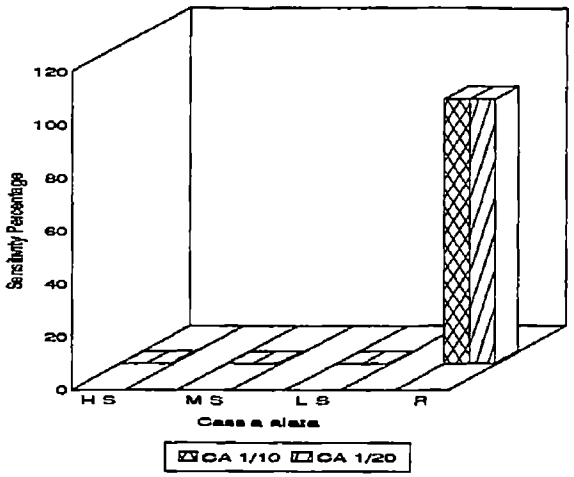
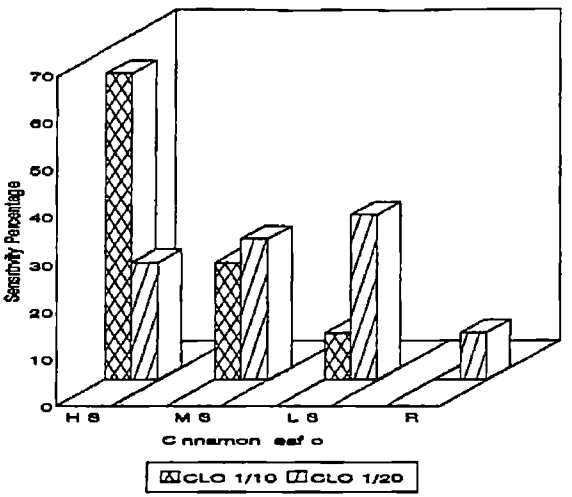
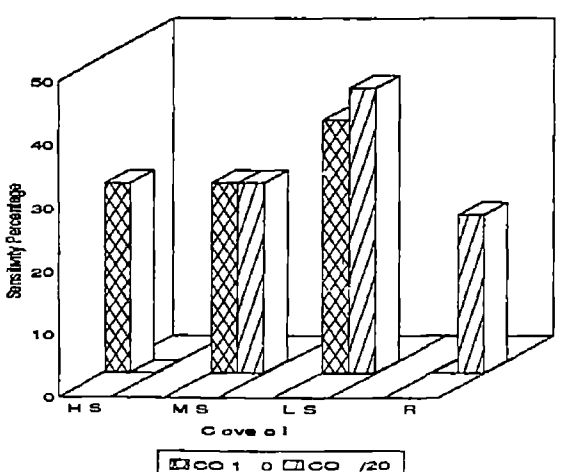


Fig 3 SENSITIVITY PATTERN OF YEAST AND YEAST LIKE FUNGAL ISOLATES AGAINST PLANT EXTRACT/ESSENTIAL OILS







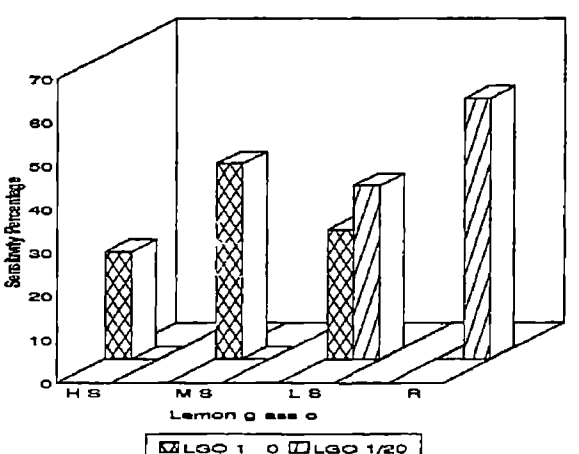


Plate 1. *C. tropicalis*: **Convex, rough and discrete colonies**

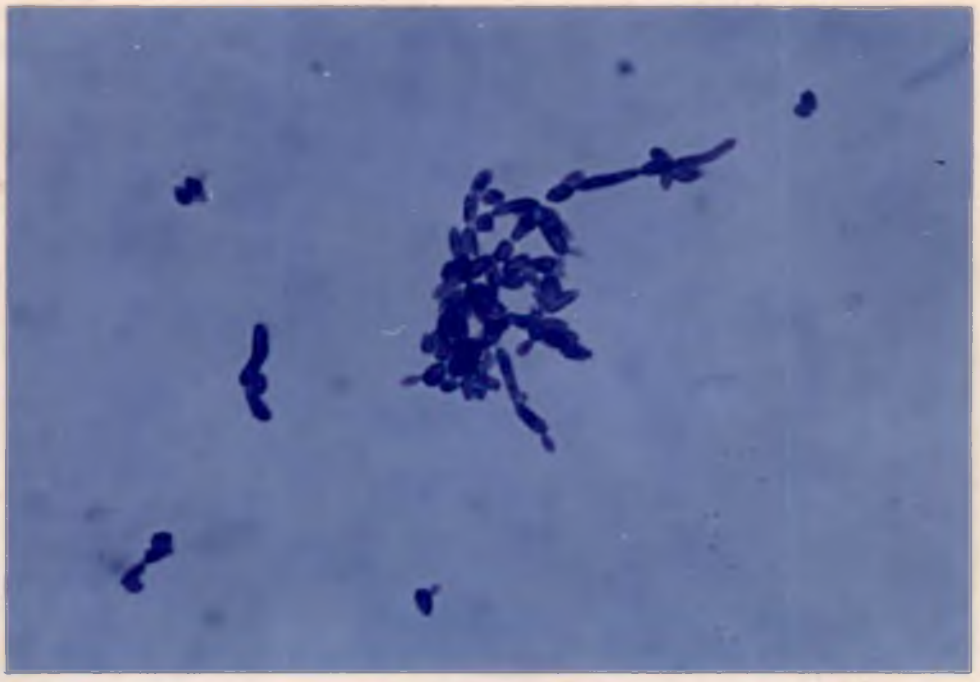


Plate 2. *C. tropicalis*: **Blastospores singly or in groups all along the pseudohyphae.**
Gram's stain x 1000

Plate 3. ***C. parapsilosis*****: Smooth, creamy and convex type colonies.**

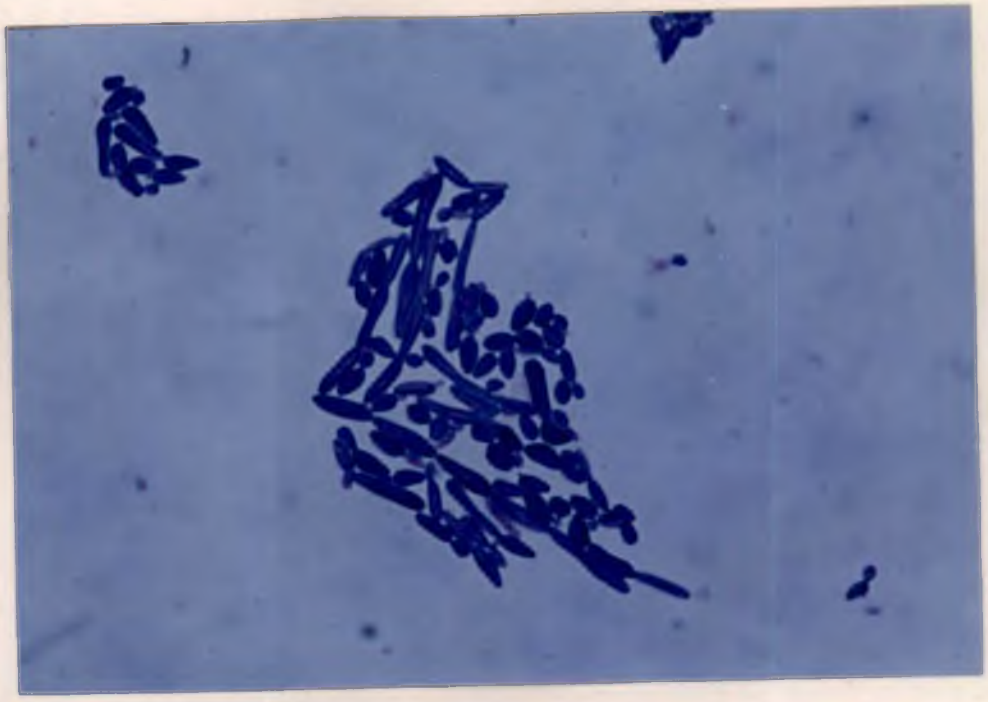


Plate 4. ***C. parapsilosis*****: Blastospores singly or in small clusters are seen along the pseudohyphae. Gram's stain x 1000**

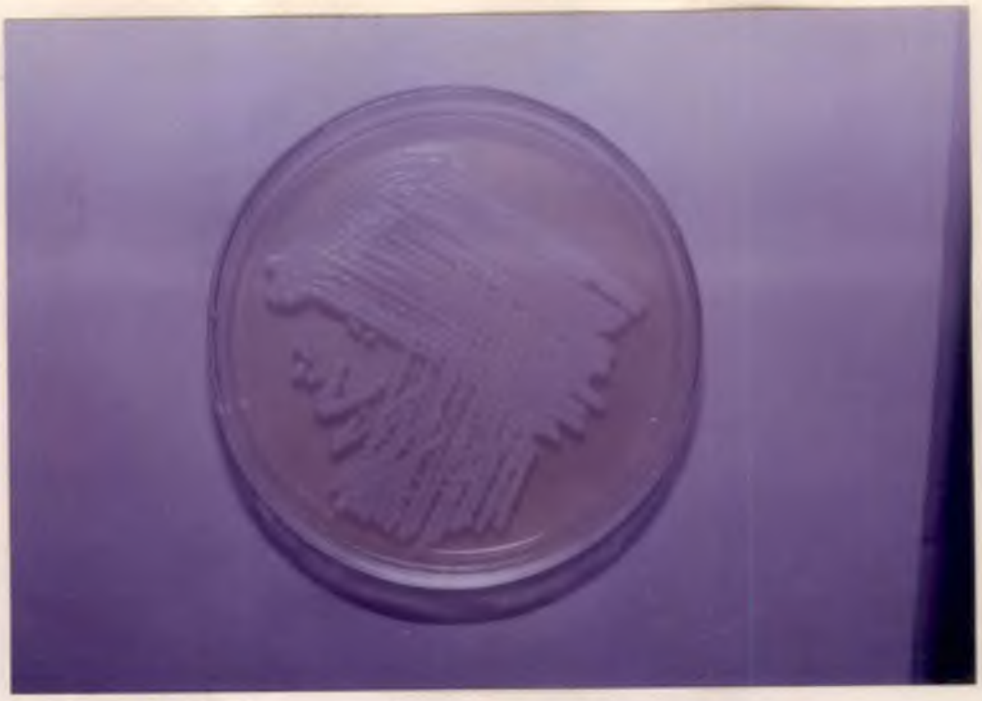


Plate 5. *C. guillermondii*: **Granular, convex and creamy type colonies.**

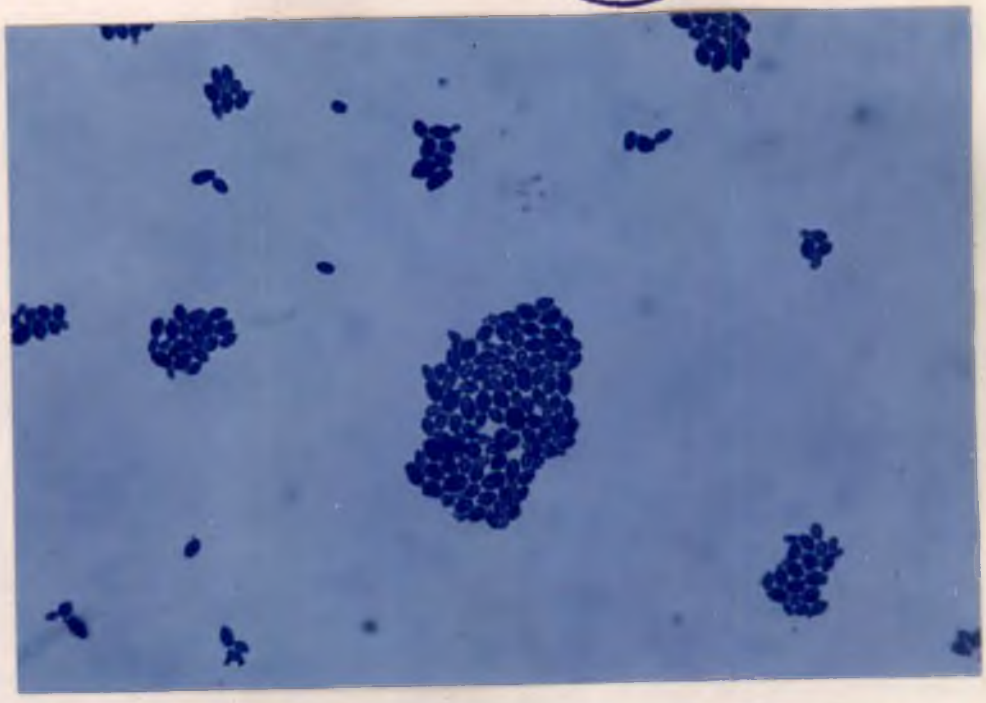


Plate 6. *C. guillermondii*: **Oval yeast cells. Gram's stain x 1000**

Plate 7. ***G. candidum*: Convex, spreading rough colonies**

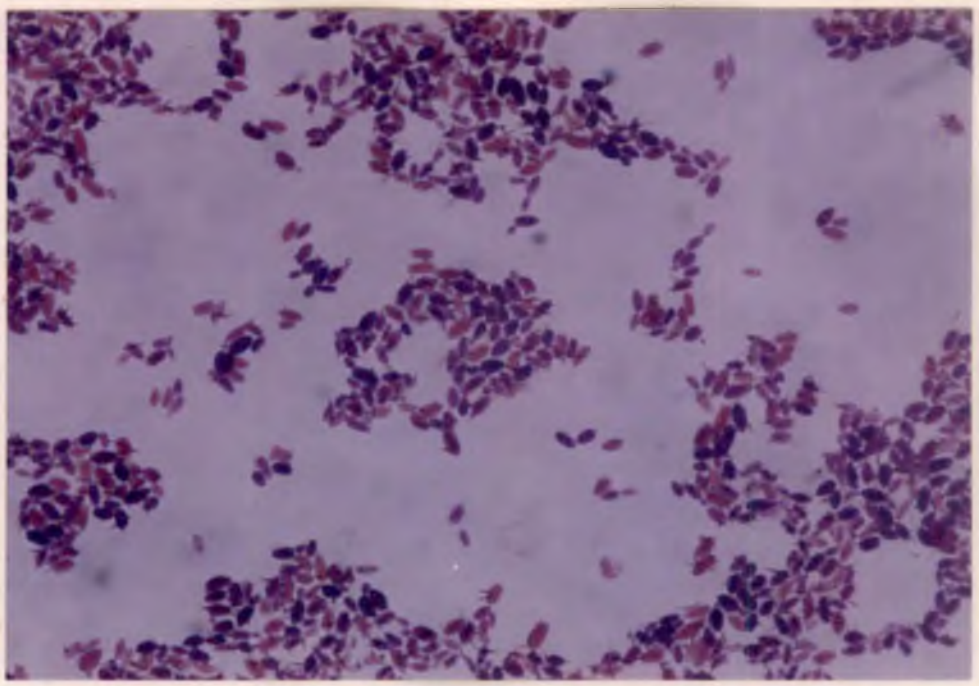


Plate 8. ***G. candidum*: Oval yeast cells and arthrospores varying in size and roundness of their ends. Gram's stain x 1000**

Plate 9. *T. cutaneum*: Convex, rough,discrete big colonies.

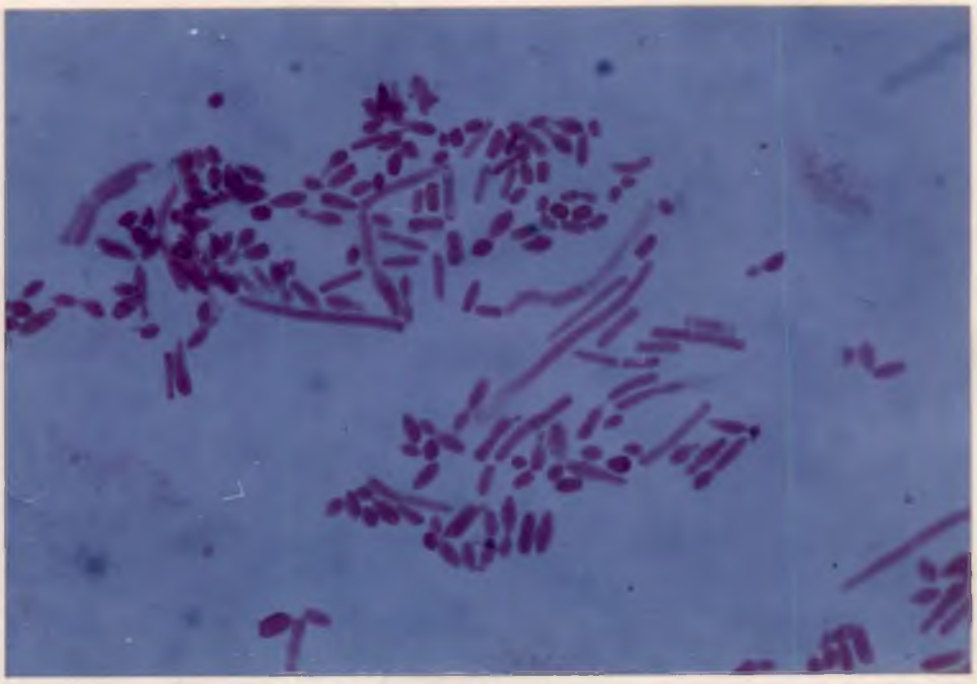


Plate 10. *T. cutaneum*: Arthrospores and blastospores. Gram's stain x 1000

Plate 11. ***S. cerevisiae*****: Rough mucilaginous colonies.**

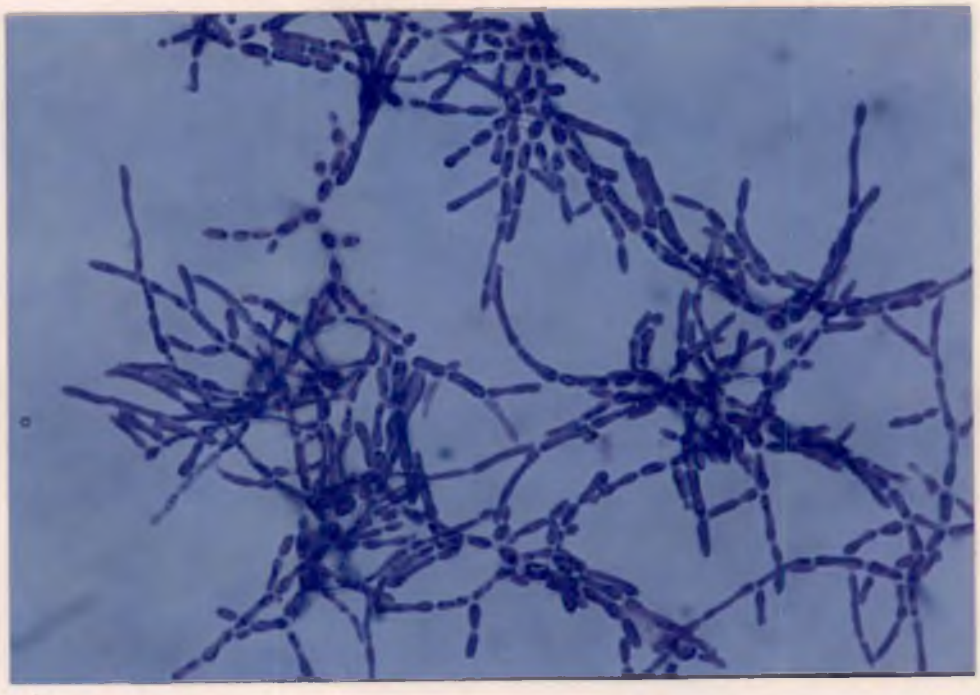


Plate 12. ***S. cerevisiae*****: Yeast cells of varying shapes with multilateral budding.**
Gram's stain x 1000

Plate 13. Torulopsis spp: Granular, convex creamy, very discrete and piliform type colonies.

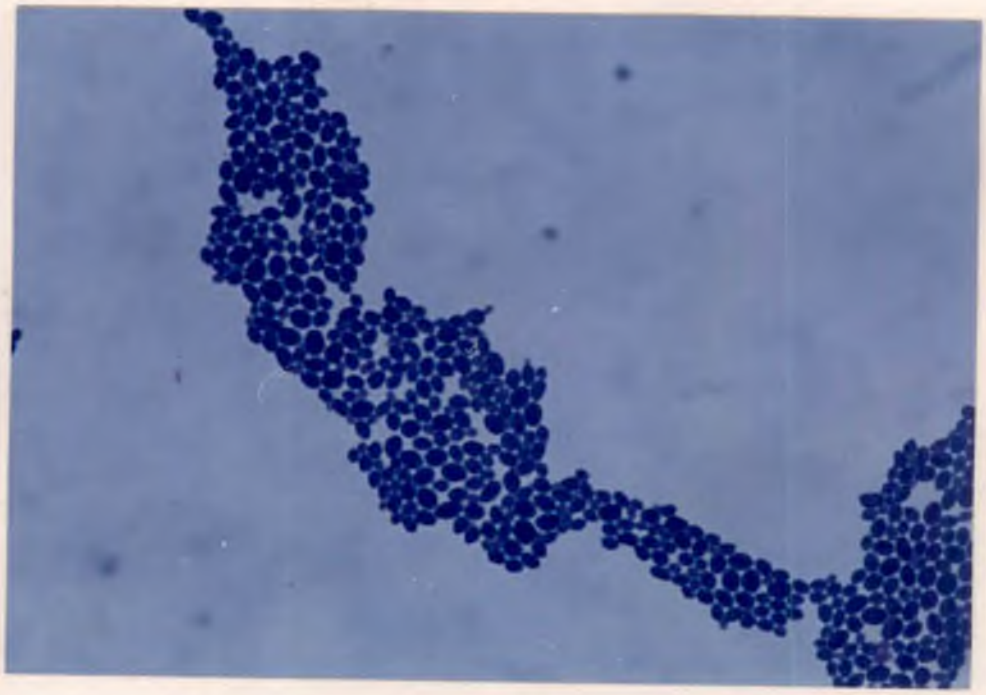


Plate 14. Torulopsis spp: Oval, single terminal budding yeast cells. Gram's stain x 1000

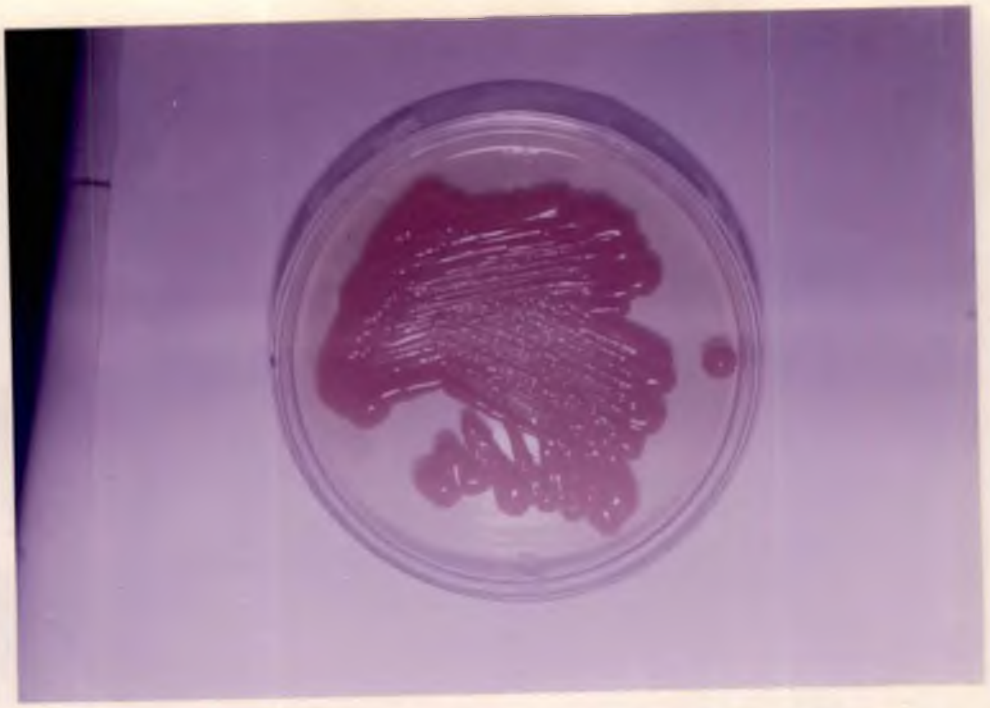


Plate 15. *R. rubra*: Soft, smooth, pink pigmented and piliform type colonies.

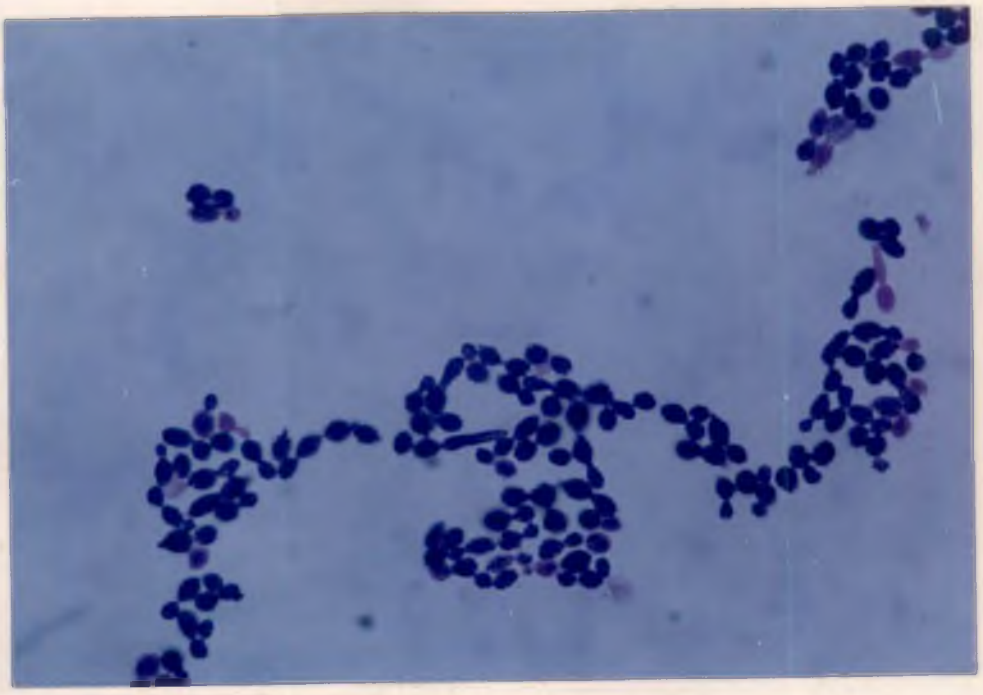


Plate 16. *R. rubra*: Round or oval budding yeast cells and rudimentary pseudohyphae. Gram's stain x 1000

Plate 17. Sepedonium spp: White and waxy type colonies. LPCB stain x 250

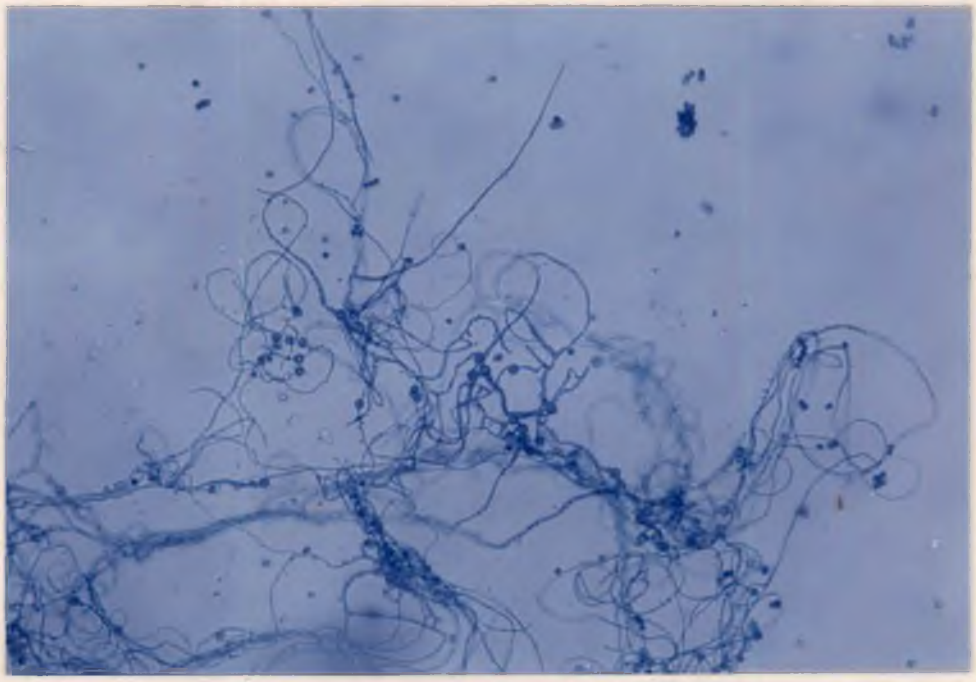


Plate 18. Sepedonium spp: Round conidia. LPCB stain x 250

Plate 19. Penicillium spp: Bluish green surface with white border colonies

Plate 20. Penicillium spp: Reverse (light) pigmentation.

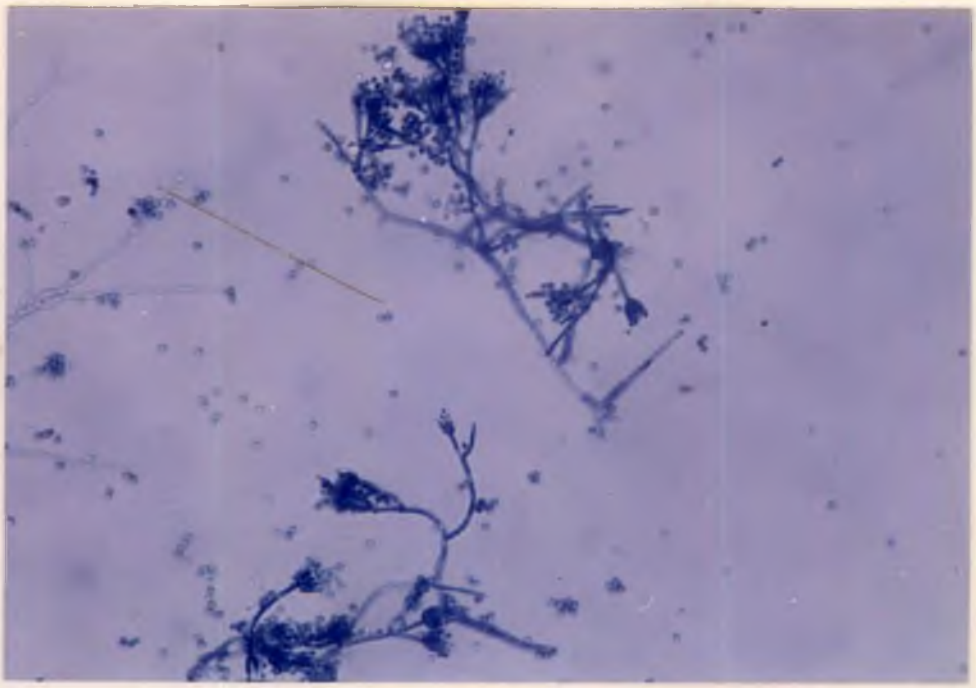


Plate 21. Penicillium spp: Conidiophore and conidia. LPCB stain x 400

Plate 22. *C. carrionii*: Dark surface, flat with slightly raised center, covered with velvetty grey green short napped mycelium.

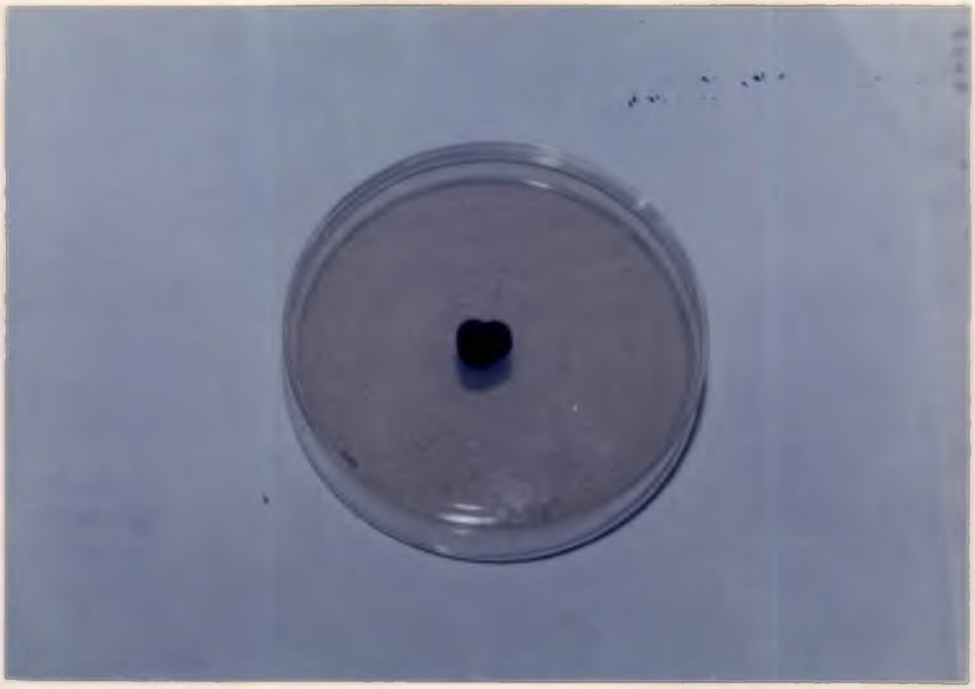


Plate 23. ***C. carrionii*: Reverse black pigmentation.**

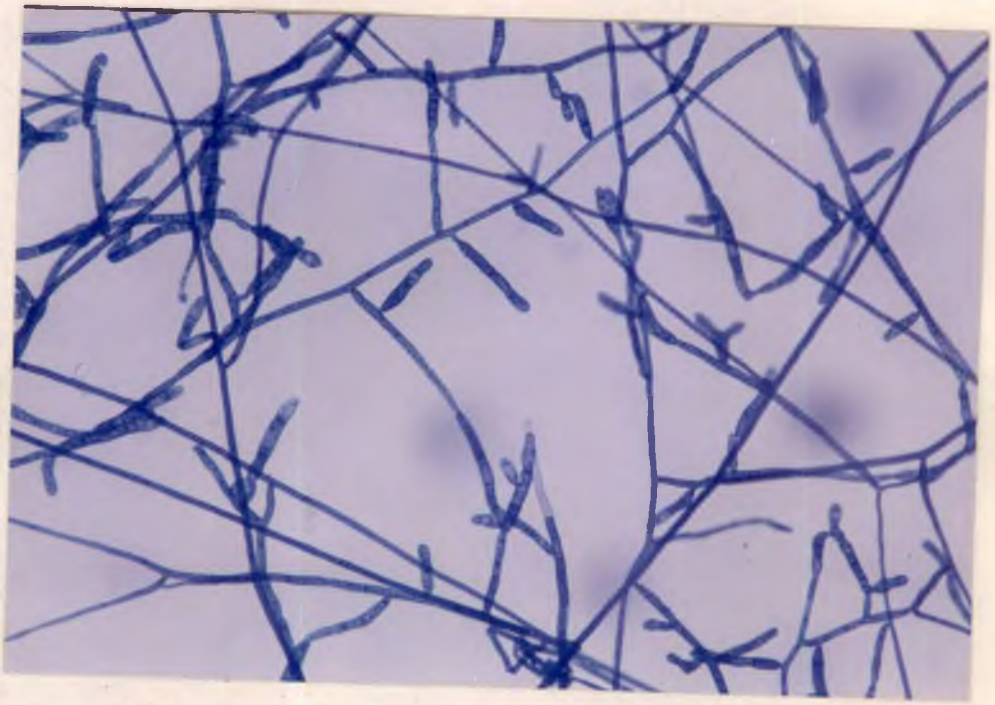


Plate 24. ***C. carrionii*: Long chains of conidia.**
LPCB stain x 250

Plate 25. *A. ochraceous* group: Spreading white, cottony aerial mycelium turning grey colour.

Plate 26. *A. ochraceous* group: Reverse grey pigmentation.

Plate 27. ***A. ochraceous*** **group: Simple long condiophore bearing globose vesicle and some what elongate sterigmata and dense aggregates of conidia. LPCB stain x 250.**

Plate 28. ***T. verrucosum*****: Small, heaped button like colony.**

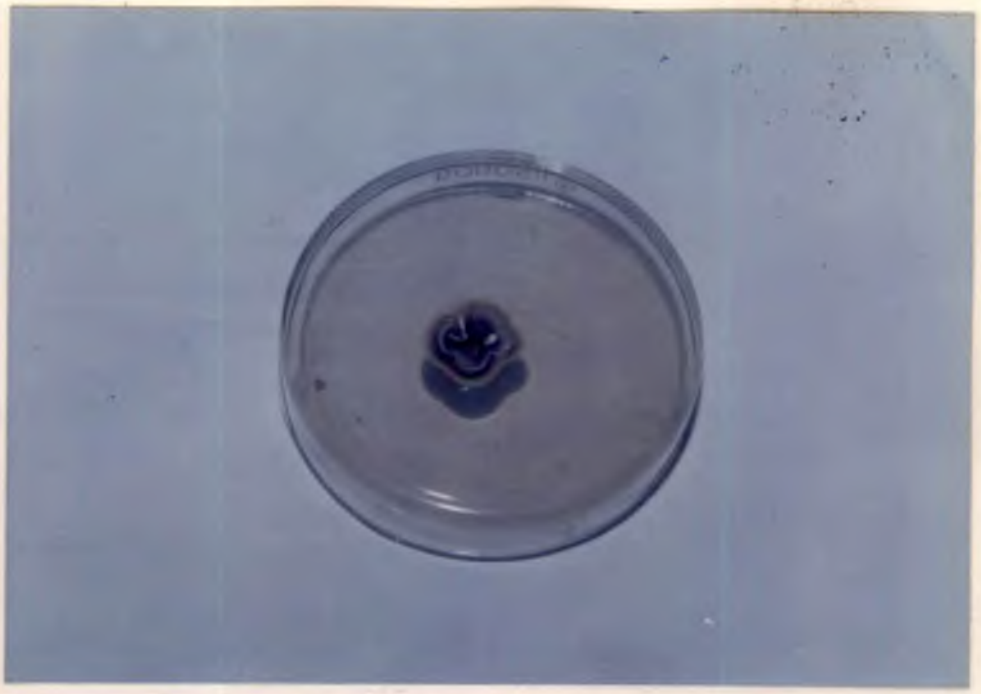


Plate 29. *T. verrucosum*: Reverse brown to yellow pigmentation.

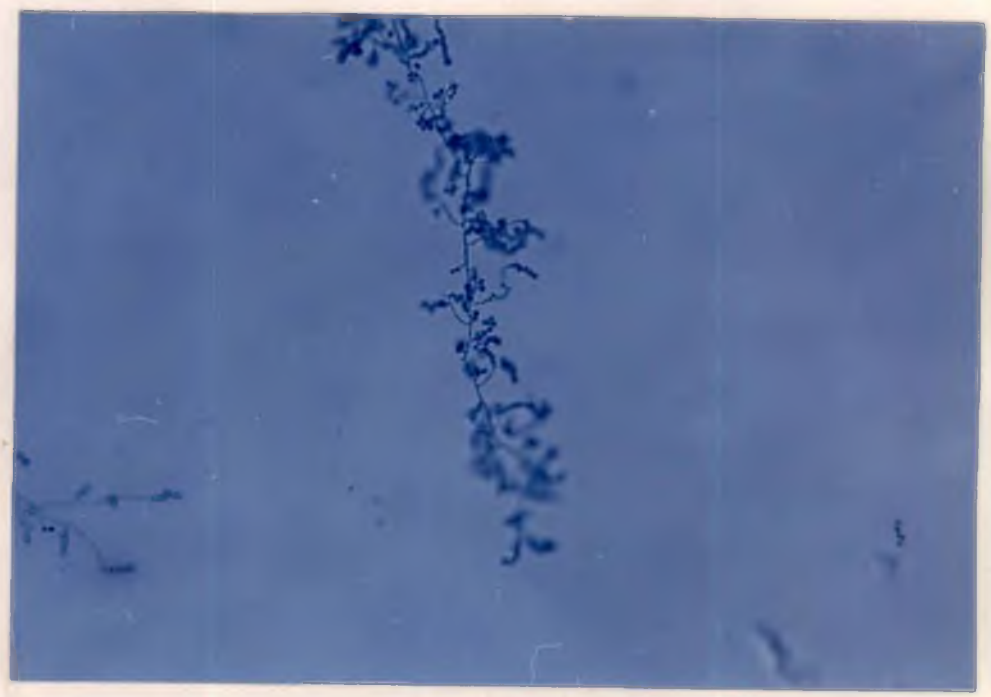


Plate 30. *T. verrucosum*: Hyphae with chlamydospores (in chains)and some antler like branches. LPCB stain x 250.

Plate 31. Penicillium spp: Greenish surface with white border.

Plate 32. Penicillium spp: Reverse (light) pigmentation.

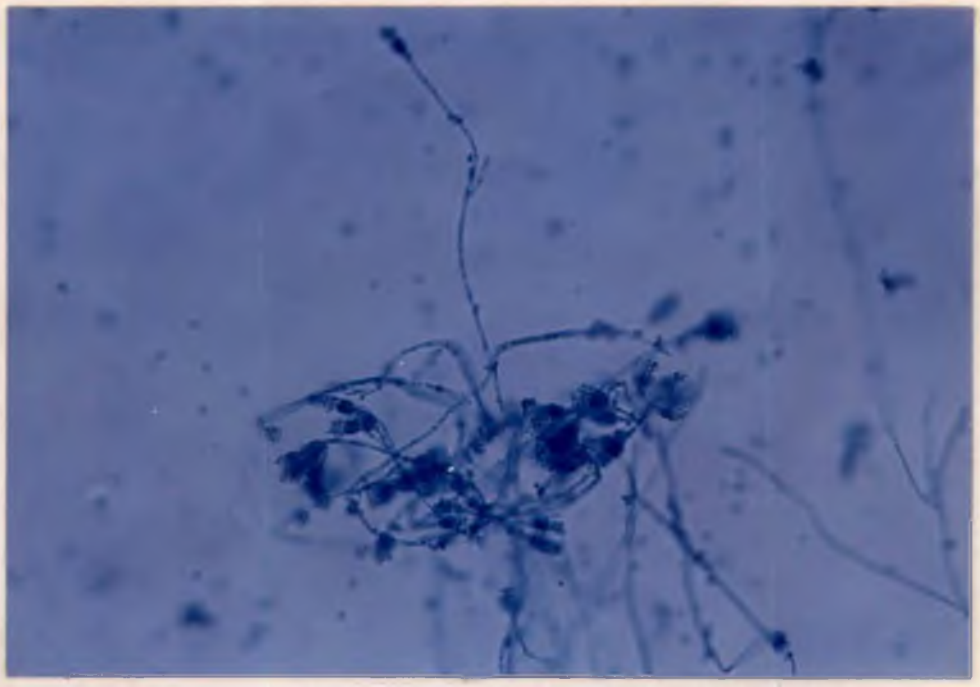


Plate 33. Penicillium spp: Conidiophore and conidia.
LPCB stain x 400.

Discussion

DISCUSSION

In the present study milk samples collected were subjected to cultural screening in order to ascertain the association of pathogenic fungi and bacteria in the causation of mastitis A total of 200 samples from 161 cows were screened Out of these 200 samples 26 samples yielded fungal agents 121 samples were positive for bacteria and the remaining 53 were negative for fungal as well as bacterial agents

Out of the 200 samples collected from the clinical mastitis cases 17 (8 5%) samples were found to have been caused by fungi and nine (4.5%) were identified to be mixed infection of fungi as well as bacteria The incidence of mycotic mastitis was found to be 13 per cent Several other workers had reported varying percentages of incidence of fungal mastitis Higher rates of incidence 29 27 per cent and 34 per cent were reported by Simaria and Dholakia (1986) Sudarwanto (1987) respectively Likewise lower incidence of mycotic mastitis (6 7%) was reported by Monga and Kalra (1971) Farnsworth and Sorensen (1972) had recorded two per cent while Sharma *et al* (1977) observed 4 35 per cent Awad *et al* (1980) Singh *et al* (1992) and Daljeet chhabra *et al* (1996) had documented the percentage incidence as 6 1 8 51 and eight respectively

The relatively higher incidence of fungal mastitis in this study may be due to the extensive and indiscriminate use of antibiotics for treatment of mastitis because some of the samples were collected from the mastitis cases which had been treated with antibiotics for the same

Neither bacteria nor fungal agents could be isolated from 26 5 per cent of the milk samples This may be due to the fact that the animals might have undergone treatment with heavy dose of antibiotics before the collection of samples The antibiotic therapy might have totally knocked out the organisms This may be the reason for not getting any organism in some of the milk samples

In the present study 4 5 per cent of the milk samples gave bacteria and fungi Misra and Panda (1986) isolated both bacteria and fungi from 20 per cent of the cases Primarily the mastitis is caused by bacteria for which a full course of antibiotic therapy should be undertaken Thus an inadequate antibiotic therapy may not destroy the entire pathogenic bacteria causing mastitis during which time the fungi may take a secondary role along with undestroyed bacteria Both bacteria and fungi in mastitis milk samples had been encountered by Stuart (1951) and Misra and Panda (1986)

Secondly fungi contaminated antibacterial preparations used for treatment of mastitis may be another possible reason for the dual role played by fungi and bacteria in the causation of mastitis (Farnsworth 1977)

This study was conducted during the period from April 1995 to January 1996 Most of the fungi isolated were obtained from the milk samples during June to November In this period the mean temperature was less than 27°C and relative humidity was greater than 75 per cent This is very conducive for fungus growth Similar findings were encountered by Sudharma *et al* (1985) Sharma (1983) in an attempt to find out the seasonal influence of fungal mastitis reported that the prevalence of mycotic mastitis was highest in winter and lowest in summer

Isolation

Among the 26 isolates 10 belonged to genus Candida and four to *G candidum* three *T cutaneum* one *S cerevisiae* one Torulopsis spp one *R. rubra* and six isolates were moulds The yeasts isolated were *C tropicalis* (7) *C parapsilosis* (2) *C guilliermondii* (1) *G candidum* (4) *T cutaneum* (3) *S cerevisiae* (1) Torulopsis spp (1) and *R rubra* (1) In clinical milk samples Candida alone contributed 38 per cent of the total fungal isolates This finding is more or less similar to the findings of Gupta *et al* (1981) who reported 38 per cent candida isolates from clinical milk samples However Prasad and Prasad (1966) Monga and Kalra (1971) Jand and Dhillon

(1975) Simaria and Dholakia (1986) reported relatively higher percentage of candida isolates from milk samples

The major pathogen isolated was *C tropicalis* which constituted 27 per cent Loken *et al* (1959) Richard *et al* (1980) Gupta *et al* (1981) and Simaria and Dholakia (1986) reported relatively more frequent isolation of *C tropicalis* from mastitis udders than any other species of candida

C tropicalis is not generally considered pathogenic for man and can quite frequently be isolated from normal individuals who may act as the source of infection (Benham 1957)

C parapsilosis was encountered twice from clinical samples Reports of the isolation of *C parapsilosis* included those of Monga and Kalra (1971) Richard *et al* (1980) Gupta *et al* (1981) Yeo and Choi (1982) Tanwani and Yadawa (1983) Sakurai *et al* (1986) Simaria and Dholakia (1986) Dhabali singh *et al* (1989) Singh *et al* (1992) Gosch (1993) and Kuo and Chang (1993)

C guilliermondii was isolated only once from clinical milk samples It was isolated from clinical mastitis milk samples by Singh and Singh (1968) Farnsworth and Sorensen (1972) Monga and Kalra (1971) Jand and Dhillon (1975) Kadic *et al* (1983) Deutz and Kuttin (1990) Gosch (1993) and Kuo and Chang (1993)

S cerevisiae could be isolated from one sample Reports of isolation of Saccharomyces spp by Awad *et al* (1980) Kadic *et al* (1983) Kuo and Chang (1993) were available in the relevant literature

Reports of isolation of Torulopsis spp from mastitis milk sample by Sakurai *et al* (1986) and Kuo and Chang (1993) proved the association of this organism in the causation of mastitis In this study also one strain of Torulopsis was isolated in one case

It was interesting to note that *G candidum* was isolated from all the four quarters of the same animal Jand and Dhillon (1975) Misra and Panda (1986) Costa *et al* (1993) isolated *G candidum* from mastitis milk samples *G candidum* is isolated frequently from the sputum skin and feces of patients without clinical disease So the cow might have contracted the infection from the milking man

T cutaneum was isolated from three mastitis milk samples of three animals Reports of isolation of *T cutaneum* include those of Saito *et al* (1980) Kadic *et al* (1983) Sharma (1983) and Costa *et al* (1993) Trichosporon is common in air soil body surface and stagnant waters (Kirk and Bartlett 1986) So unsanitary conditions and poor management of animals may be the reason for Trichosporon infection in animals

R rubra was isolated only from one case of mastitis milk sample Rhodotorula spp was isolated from mastitis milk sample by Jand and Dhillon (1975) Simaria and Dholakia (1986) Awad *et al* (1980) Sakurai *et al* (1986) and Deutz and Kuttin (1990)

Involvement of two different fungal agents *C tropicalis* and *C guilliermondii* was evidenced by isolation of these agents from two quarters of same animal In another case two different genera *C parapsilosis* and *R rubra* were isolated from two quarters This indicates that multiple fungal infection could occur in animals as the case of multiple bacterial infection

Six mould (3%) were isolated from 200 clinical milk samples examined These were Sepedonium spp *Aspergillus ochraceous* group *Cladosporium carrionii* Penicillium spp (2) and *Trichophyton verrucosum*

Aspergillus spp was isolated from milk sample by Singh and Sirgh (1968) Jand and Dhillon (1975) Thompson *et al* (1978) Matsuoka *et al* (1981) Misra and Panda (1986) Dhabali singh *et al* (1989) Marcos *et al* (1990) Singh *et al* (1992) and Reddy and Khan (1994)

Sharma *et al* (1977) isolated Sepedonium spp from cows suffering from subclinical mastitis

Cladosporium spp was isolated from mastitis milk sample by Sharma (1983) Dhabali singh *et al* (1989) and Singh *et al* (1992)

Penicillium spp was isolated from mastitis milk sample by Sharma (1983) Misra and Panda (1986) Cuci and Matraku (1987) Mehrotra and Rawat (1989) Costa *et al* (1993) and Reddy and Khan (1994)

Trichophyton spp was isolated from mastitis milk sample by Farnsworth and Sorensen (1972) *T verrucosum* is the causative agent for ring worm infection in animals It will produce the lesion in the superficial part of the body In this study only one milk sample yielded *T verrucosum* Trichophyton spp induced mastitis was reported by Murphy and Drake (1947) and they succeeded in reproducing the condition experimentally

The low incidence of mastitis caused by the moulds may be due to the fact that the yeasts by virtue of their ability to break up into small vegetative reproducing units can adapt themselves in the ducts and acini of the udder like bacteria while the moulds cannot as suggested by Ainsworth and Austwick (1959)

The high incidence of candidial mastitis may be due to the ability of the genus to utilize penicillin and tetracycline as a source of nitrogen for their growth and these antibiotics are commonly employed in mastitis therapy (Mehnert *et al* 1964) Fungi are not inhibited by antibacterial antibiotics rather they provide the suitable condition for selective growth by destroying the bacteria present in the udder (Rahman and Baxi 1983)

The isolation of fungi post antibiotic treatment suggests that they are not only resistant to such antibiotics but produce disease in the absence of fast multiplying bacterial competitors or by utilizing antibiotics for their basic growth requirements (Mehnert *et al* 1964)

Besides yeast contaminated antibiotic preparations used for udder infusion may result in secondary infection of mammary gland (Farnsworth 1977) Probably these could be the reasons for increased incidence of mycotic mastitis in recent years

Trauma of udder improper and incomplete milking improper use of milking machines abnormalities of teat and udder improper feeding high milk yield early lactation, hereditary factors old age unsanitary conditions and poor management were said to be predisposing factors for bovine fungal mastitis (Henning 1956) Trauma from the milking machine and use of irritant teat dips may predispose the gland to yeast infection (Giesecke *et al* 1968)

Yeast may be disseminated by certain species of birds (Montovani *et al* 1970) Yeast are saprophytic on plant and plant products and are normal inhabitants of skin of the udder and teats where they exist in low numbers (Loftsgard and Linquist 1960)

Fungi are opportunistic and ubiquitous in nature and may lead to the hazards for the udder of animals under certain condition of lowered resistance of animals (Jand and Dhillon 1975)

Identification

Yeast and yeast like fungi

Seven isolates produced similar reactions to that of *C tropicalis* except in fermentation of maltose and sucrose Five isolates produced only acid and no gas in maltose and sucrose fermentation

Three isolates produced biochemical reactions similar to *T cutaneum* but one of these isolates could produce acid by maltose fermentation

Two isolates showed similar reactions to that of *C parapsilosis* except in sucrose fermentation In sucrose fermentation these two produced acid only

Yet another isolate exhibited reactions similar to that of *C guilliermondii* except in sucrose and galactose fermentation These types of variations in one or two characters from the standard properties of the organism have been observed by Wolf *et al* (1975)

Organism isolated versus changes in the udder and milk

In 75 per cent of the cases *C tropicalis* organism produced chronic mastitis characterised by mild fibrosis of udder decreased milk yield and straw yellow coloured milk with flakes These changes are in agreement with the findings of Loken *et al* (1959)

Mastitis due to *T cutaneum* was manifested by swelling of the gland as reported by Murphy and Drake (1947)

G candidum produced chronic mastitis with reduction in milk yield and the milk was watery with clots There is no report regarding the udder changes due to *G candidum* infection

C parapsilosis mastitis was chronic in nature with flakes noticed in the milk Mastitis caused by *C guilliermondii* was chronic type and mild fibrosis of udder was noticed No reports regarding the changes in milk sample from cases of mastitis caused by *C parapsilosis* and *C guilliermondii* are available

In mastitis induced by *S cerevisiae R rubra* and Torulopsis spp flakes were present in the milk but reports to support this observations could not be traced in the concerned literature

In majority of the cases mould produced chronic mastitis characterized by hardness of udder with reduction in milk yield and straw yellow coloured milk viscid in consistency

However the changes in udder and milk characteristics are not very pathognomonic to make differential diagnosis between and fungal and bacterial mastitis

Antifungal susceptibility test

Antifungal chemotherapeutic agent

In vitro antifungal chemotherapeutic sensitivity tests were carried out on all the 26 fungal isolates by disc diffusion method described by Bauer *et al* (1966)

The most preponderant yeast and yeast like fungal organism isolated was *C tropicalis* All the isolates were sensitive to all the antifungal agents tested except Griseofulvin It showed high degree of sensitivity to Itraconazole Ketoconazole and Pimaricin The degree of sensitivity was found to decrease with the higher dilutions of these antifungal agents Of these isolates 57 per cent showed resistance to Fluconazole So Itraconazole Ketoconazole Pimaricin Amphotericin B Clotrimazole and Nystatin could be used effectively for the treatment of *C tropicalis* mastitis

The second candida spp isolated was *C parapsilosis* It showed a high degree of sensitivity to Clotrimazole Nystatin Itraconazole Ketoconazole and Pimaricin and medium sensitivity to Amphotericin B Both the isolates showed resistance to Fluconazole So it was noted that the above said seven antifungal agents could be used for the treatment of *C parapsilosis* mastitis

The third spp of candida isolated was *C guilliermondii* It showed high degree of sensitivity to Clotrimazole Itraconazole Ketoconazole and Pimaricin moderate sensitivity to Amphotericin B and Nystatin and resistance to Fluconazole

In vitro studies by Lipnicki *et al* (1975) and Jand *et al* (1978) revealed the sensitivity of *C tropicalis C parapsilosis* and *C guillermondii* to Nystatin Hartmann and Kilchsperger (1982) studied the 19 candida spp isolated from the milk and found that all the strains were inhibited by Natamycin at or above 6 25 ug/ml Bansal *et al* (1989) tested the antifungal sensitivity of *C guillermondii* and *C parapsilosis* to five antimycotic drugs and revealed that Clotrimazole (5 ug/disc) and Ketoconazole (10 ug/disc) were highly effective drugs at this concentration

All the candida species isolated were sensitive at varying degree to all the antifungal agents used for the study except Fluconazole All the isolates showed higher inhibitory zone to Itraconazole So Itraconazole has been found to be the drug of choice followed by Ketoconazole Pimaricin Clotrimazole Nystatin and Amphotericin B for the treatment of candidial mastitis All the isolates showed resistance to Griseofulvin Similar finding was encountered by Jand *et al* (1978) and Bansal *et al* (1989)

The organism under the genus Geotrichum was *G candidum* It showed high degree of sensitivity to Clotrimazole Nystatin Itraconazole Ketoconazole and moderate sensitivity to Amphotericin B It showed resistance to Fluconazole Griseofulvin and Pimaricin

The isolates under the genus Trichosporon were speciated as *T cutaneum* It showed high degree of sensitivity to Clotrimazole and Ketoconazole and moderate sensitivity to Amphotericin B Nystatin and Pimaricin Thirty three per cent of the isolates showed resistance to Itraconazole and 100 per cent to Griseofulvin Minagawa *et al* (1982) reported that the Ketoconazole had the MIC value of 0 625 ug\ml against Trichosporon spp

S cerevisiae was highly sensitive to Amphotericin B Clotrimazole Nystatin Itraconazole and Ketoconazole and moderately sensitive to Pimaricin It showed resistance to Fluconazole and Griseofulvin Higher inhibitory zone was shown by Nystatin It was

found that Torulopsis spp was highly sensitive to Clotrimazole Nystatin Itraconazole and Ketoconazole and moderately sensitive to Amphotericin B and Pimaricin It was resistant to Fluconazole and Griseofulvin All the three concentrations of Itraconazole had shown high sensitivity against Torulopsis The higher inhibitory zone was given by Itraconazole

R rubra was found to be highly sensitive to Itraconazole Ketoconazole and Pimaricin and moderately sensitive to Clotrimazole and Nystatin All the three concentrations of Pimaricin showed higher sensitivity but the lower concentration of Ketoconazole and Itraconazole showed medium sensitivity It was resistant to Fluconazole and Griseofulvin Pimaricin showed higher zone of inhibition

In vitro antifungal susceptibility of yeast and yeast like fungal isolates showed that Itraconazole was the drugof choice followed by Ketoconazole Pimaricin Clotrimazole Nystatin and Amphotericin B Most of the isolates showed resistance to Fluconazole This may be due to the lower concentration of Fluconazole If the concentration had increased it might have given a higher degree of inhibition Griseofulvin had no effect on yeast and yeast like fungal organism even in higher concentrations Similar findings were reported by Jand *et al* (1978) and Bansal *et al* (1989)

In vitro antifungal susceptibility of mould isolates showed that Clotrimazole was the drug of choice followed by Itraconazole Ketoconazole and Pimaricin Less sensitivity was shown aga nst Amphotericin B and Nystatin All the mould isolates showed resistance to Fluconazole and Griseofulvin

Antifungal activity of plant extracts

The antifungal property of the alcoholic and aqueous extracts of *Cassia alata* and essential oils of three plant was assessed by disc diffusion method described by Bauer *et al* (1966)

In the present study aqueous and methanol extract of *Cassia alata* revealed no antifungal property eventhough Fuzellier *et al* (1982)had reported the antifungal property of the aqueous extract from *Cassia alata* leaves

Essential oils extracted from cinnamon clove and lemon grass exhibited broad spectrum *in vitro* inhibitory activity against yeast and yeast like fungal organisms Because of the ability of these medicinal plants and their products to inhibit the growth of pathogenic microorganisms they have been used in the treatment of infectious diseases

Out of the three essential oils cinnamon leaf oil was found to be highly effective against all the yeast and yeast like fungal organism Similar antifungal studies on essential oils obtained from cinnamon leaf clove and lemon grass against animal pathogenic fungi had been reported by Frazier (1967) Onawunmi (1989) and Sudha devi and Pillai (1993)

It was interesting to note that cinnamon leaf oil at 1 in 10 dilution showed more *in vitro* antifungal activity to all the yeast and yeast like fungal organisms compared to Amphotericin B Fluconazole and Griseofulvin On comparison to Pimaricin in its antifungal effect other than *C parapsilosis* and R rubra cinnamon oil at 1 in 10 dilution could produce more diameter zone of inhibition for the rest of the yeast and yeast like fungi *G candidum* was found to be more amenable to cinnamon leaf oil than all the commercial antifungal agents except Nystatin

The results accrued out of the present study points to the possibility of employing cinnamon leaf oil at 1 in 10 dilution as a drug more efficient than the commonly available antifungal agents to combat fungal mastitis The antifungal activity of essential oils of cinnamon had been reported by Frazier (1967) Bullerman (1974) Yousef *et al* (1978) Saksena (1984) and Thompson and Cannon (1986)

The essential oil obtained from clove at 1 in 10 dilution was able to suppress the growth of yeast and yeast like fungi such as *G candidum R rubra* and *C guilliermondii* Sudha devi and Pillai (1993) reported the inhibition of *C guilliermondii* and *T cutaneum* by the essential oil of clove Comparative evaluation of the efficacy of this oil with common antifungal agents revealed that this oil was more powerful than Amphotericin B Clotrimazole Nystatin and Itraconazole to inhibit *R rubra* It was also found to be more effective in suppressing the growth of fungal organism than Fluconazole and Griseofulvin

The essential oil of lemon grass showed remarkable antifungal property to all the organisms tested Similar antifungal property have been reported by Onawunmi (1989) *In vitro* studies indicated that in combating *G candidum* it was found to be more effective than Amphotericin B Fluconazole and Pimaricin at 1 in 10 dilution In the suppression of *R rubra* this oil was found superior to Amphotericin B and Clotrimazole Eventhough it has good suppressive action on *G candidum* and *R rubra* at 1 in 10 dilution some antifungal agents were superior to this oil

Cinnamon leaf oil at 1 10 dilution showed more *in vitro* antifungal activity to all the mould isolates compared to Amphotericin B Fluconazole Griseofulvin Ketoconazole and Nystatin On comparison to Pimaricin in its antifungal effect CLO at 1 10 dilution could produce inhibition to Penicillium spp and when compared to Itraconazole it showed inhibition to *A ochraceous* group Comparison to Clotrimazole it showed inhibition to *C Carronii*

Clove oil at 1 10 dilution was able to suppress the growth of Sepedonium spp and *C carronii* It was found to be more effective in suppressing the growth of mould than Fluconazole Griseofulvin and Nystatin

Lemon grass oil at 1 10 and 1 20 dilutions revealed no antifungal activity against mould

The antifungal effect of these three essential oils against various pathogenic fungi had been reported by Bullerman (1974) Yousef *et al* (1978) Thompson and Cannon (1986) Onawunmi (1989) Mangiarotti *et al* (1990) Shadab qamar *et al* (1992) and Zhang (1995)

Though all the essential oils tested in this study possess varying degree of antifungal property further in vivo studies are required in order o prove its efficacy to combat natural/experimental infections before its utility is advocated for field applications

Summary

SUMMARY

A total of 200 milk samples from clinical cases of bovine mastitis were collected and subjected to cultural screening Pathogenic micro organisms could be isolated from 147 samples Fungi were isolated from 26 samples and bacteria from 121 samples fifty three samples were negative for fungal as well as bacterial agents

The yeast and yeast like fungal isolates were identi-fied based on their colony characters microscopic morphology growth at 25°C with cycloheximide presence or absence of capsule growth on corn meal agar containing Tween 80 germ tube test urease test at 25°C growth at 37°C on Sabouraud s dextrose agar growth n Sabouraud s dextrose broth fermentation of sugars(dextrose maltose sucrose lactose and galactose) and sugar assimilation test employing dextrose maltose sucrose lactose melibiose cellobiose mositol xylose raffinose trehalose and dulcitol

The filamentous moulds isolated from milk samples were identified based on the rate of growth general topography texture surface pigmentation reverse pigmentation and microscopic examination

The major yeast and yeast like fungi isolated were candida spp namely *C tropicalis* (27%) *C parapsilosis* (7 69%) and *C guillermondii* (3 84%) The other organisms were *G candidum* (15 38%) *T cutaneum* (11 53%) *S cerevisiae* (3 84%) Torulopsis spp (3 84%) and *R rubra* (3 84%)

The filamentous mould isolated were Sepedonium spp (3 84%) *Cladosporium carrionii* (3 84%) Penicillium spp (7 69%) *Trichophyton verrucosum* (3 84%) and *Aspergillus ochraceous* group (3 84%)

In majority of the cases yeast and yeast like fungi produced chronic mastitis in which hardness of udder and reduction in milk yield with watery milk and flakes were noticed

In cases of mastitis where in mould was involved produced chronic mastitis characterized by hardness of udder and reduction in milk yield with straw coloured milk viscid in consistency

Essential oil from cinnamon leaf and lemon grass was extracted by Clevenger apparatus and alkaloids of *Cassia alata* was obtained by aqueous and alcoholic extraction

Antifungal agents were purchased from local market and antifungal sensitivity discs were prepared

Sensitivity discs incorporating the essential oils\alkaloids were also prepared

In vitro antifungal activity of the above mentioned agents was assessed on the 26 fungal isolates

Yeast and yeast like fungal isolates were cent per cent sensitive to Amphotericin B (100u) Clotrimazole (10μg) Nystatin (100u) and Ketoconazole (100μg) 95 per cent sensitive to Itraconazole (0 03125μg) 80 per cent sensitive to Pimaricin (5 μg) and 30 per cent sensitive to Fluconazole (10μg) These isolates showed cent per cent resistance to Griseofulvin Itraconazole was found to give high degree of inhibition at low concentration The best antifungal drug was found to be Clotrimazole When the concentration of Itraconazole Ketoconazole and Pimaricm decreased the sensitivity of yeast and yeast like fungi also decreased

It was found that the essential oil of cinnamon leaf clove and lemon grass showed varying degree of antifungal properties

Aqueous and methanol extract of *Cassia alata* on testing failed to exhibit the antifungal activity against any of the fungal isolates

Out of the three essential oil tested cinnamon leaf oil was found to be active against all the yeast and yeast like isolates tested at 1 in 10 dilution

Dilution of 1 in 10 and 1 in 20 of cinnamon leaf oil showed inhibition of cent percent and 90 per cent of yeast and yeast like fungal isolates respectively

Essential oil from clove leaf when diluted 10 times and 20 times inhibited cent per cent and 75 per cent of yeast and yeast like fungal isolates respectively

Essential oil obtained from lemon grass (1 in 10 dilution) showed inhibitory activity against cent per cent and at 1 in 20 dilution 40 per cent of yeast and yeast like fungal isolates

Mould fungal isolates were 83 33 per cent sensitive to Amphotericin B (100u) Clotrimazole (10μg) Itraconazole (0 031μg) and Pimaricin (5μg) 50 per cent sensitive to Ketoconazole (100μg) 33 33 per cent to Nystatin (100u) 100 per cent resistant to Griseofulvin (250μg) and Fluconazole (10μg) Clotrimazole (10μg) and Itraconazole (0 031μg) were found to give high degree of inhibition followed by Pimaricin (5μg)

Griseofulvin (250μg) failed to show antifungal activity against any of the fungal isolates

Out of three essential tested *in vitro* Cinnamon leaf oil was found to be active against most of the mould isolates at 1 10 dilution

At 1 10 dilution cinnamon leaf oil showed activity against 83 3 per cent isolates and 50 per cent of the mould isolates were suppressed at 1 20 dilution

Dilution 1 10 of clove leaf oil showed inhibition of 33 3 per cent isolates and at 1 20 dilution less activity against 33 3 per cent of mould isolates was noted

Essential oil from lemon grass showed no activity against mould isolates at both 1 10 and 1 20 dilutions

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Appendix

APPENDIX

1A Assimilation media (for yeast and yeast like fungi)

Wickerham broth medium

Carbon Assimilation medium

Yeast nitrogen base	⇒	6 70 g
Appropriate carbohydrate	⇒	5 00 g
Distilled water	⇒	100 00 ml

Sterilized by Seitz filter The medium 0 5 ml quantity was added to 4 5 ml of sterile distilled water in cotton plugged tubes

1B Corn-meal agar - Tween 80

Dehydrated cornmeal Agar	⇒	21 g
Dextrose	⇒	2 g
Tween 80	⇒	10 ml
Distilled water	⇒	1000 ml

Ingredients were mixed boiled and sterilized by autoclaving at 121°C for 15 minutes Thirty to 40 ml media was poured in sterile petridishes by routine plate pouring procedure

1C. Fermentation media

(i) Beef Extract Broth

Beef Extract	⇒	3 0 g
Peptone	⇒	10 0 g
NaCl	⇒	5 0 g
Bromcresol stock solution (given below)	⇒	1 0 ml
Distilled water	⇒	1000 0 ml

Ingredients were dissolved by heating and the pH was adjusted to 7 2 Nine ml broth was placed in 16 X 125 mm cotton plugged tubes with inverted Durham s tube and sterilized by autoclaving at 121° C for 15 minutes

(ii) Brom cresol purple stock solution

Bromcresol purple	⇒	1 6 g
Ethanol 95 per cent	⇒	100 0 ml

(iii) Stock carbohydrate solution

Ten per cent aqueous solutions of dextrose maltose sucrose lactose galactose were prepared and sterilized by filtration

One ml of the stock dextrose solution was added to one tube of beef extract broth One ml of stock maltose solution was added to a second tube This process was continued for each sugar

1D. Sabouraud's Dextrose Agar (SDA)

Dextrose	⇒	40 g
Peptone	⇒	10 g
Agar	⇒	15 g
Distilled water	⇒	1000 ml

Final pH was adjusted to 5 6 and the ingredients were dissolved by boiling and sterilized by autoclaving at 10 lbs for 30 minutes

1E Sabouraud's Dextrose Broth

Commercial Sabouraud Dextrose agar (Hi media) was used

Ingredients	Grams/litre
Special peptone	⇒ 10 0
Dextrose	⇒ 20 0
Final pH (at 25°C) 5 6 ± 0 2	

Thirty gram was suspended in 1000 ml distilled water It was boiled to dissolve the medium and sterilized by autoclaving at 15 lbs pressure for 15 minutes

1F SDA with cycloheximide

Five hundred mg cycloheximide was dissolved in 10 ml of acetone and added to the boiling Sabouraud s dextrose agar It was sterilized by autoclaving at 10 lbs pressure for 30 minutes

1G Spore stain

Malachite green solution

Malachite green	⇒ 7 6 gm
Distilled water	⇒ 100 ml

Safranin solution

Safranin O	⇒ 0 25 gm
Distilled water	⇒ 100 ml

1H Tryptone Soya Agar

Ingredients		Grams/litre
Casein Enzymic Hydrolysate	⇒	15
Soya Peptone	⇒	5
Sodium Chloride	⇒	5
Agar	⇒	15
Final pH (at 25°C) 7 3 ± 0 2		

Fourty gm agar was suspended in 1000 ml distilled water and boiled to dissolve the medium completely It was sterilized by autoclaving at 15 lbs for 15 minutes

1I Urea agar

Commercial christensen urea agar base (Hi media) was used

Ingredients		Grams/litre
Peptone	⇒	1 0
Dextrose	⇒	1 0
Nacl	⇒	5 0
Disodium phosphate	⇒	1 2
Monopotassium phosphate	⇒	0 8
Phenol red	⇒	0 012
Agar	⇒	15
final pH (at 25°C) 6 8 ± 0 2		

Urea agar base of 2 4 gm was suspended in 95 ml distilled water and boiled to dissolve the medium completely Then the media was sterilized by autoclaving at 10 lbs pressure for 20 minutes Cooled to 45°C and five ml of sterile 40 per cent urea solution was aseptically added to the 95 ml base Then the media was dispensed in tubes and cooled in a slanting position

PREVALENCE OF YEAST AND YEAST LIKE FUNGI IN BOVINE MASTITIS AND THEIR *IN VITRO* DRUG SENSITIVITY

By

K SUKUMAR

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree

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KERALA AGRICULTURAL UNIVERSITY

Department of Microbiology

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY THRISSUR

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ABSTRACT

A total of 200 milk samples from clinical cases of bovine mastitis were culturally screened during a period of six months Pathogenic fungal organisms could be isolated only from 26 samples Out of this 26 positive samples yeast and yeast like fungal organisms were isolated from 20 samples and mould from six cases

The major pathogen isolated were candida spp namely *C tropicalis C parapsilosis* and *C guillermondi* The other organisms were *Geotrichum candidum Trichosporon cutaneum Saccharomyces cerevisiae* Torulopsis spp and *Rhodotorula rubra*

The filamentous fungi isolated were Sepedonium spp *Aspergillus ochraceous* group *Cladosporium carrionii* Penicillium spp and *Trichophyton verrucosum*

In majority of the cases yeast and yeast like fungi produced chronic mastitis in which hardness of udder and reduction in milk yield with watery milk containing flakes were noticed

In cases of mastitis where in mould was involved chronic mastitis characterized by hardness of udder and reduction in milk yield with straw yellow coloured milk, viscid in consistency

Sensitivity pattern of the fungal isolates to the commonly employed antifungal chemotherapeutic agents like Amphotericin B Clotrimazole Fluconazole Griseofulvin, Itraconazole Ketocanazole Nystatin and Pimaricin (Natamycin) was elucidated Among the above agents Clotrimazole and Itraconazole exhibited maximum inhibitory activity All the isolates were found to be resistant to Griseofulvin

In vitro drug sensitivity pattern of fungal isolates employing the discs impregnated with essential oils of cinnamon, clove and lemon grass and alkaloids of *Cassia alata* was studied Cinnamon leaf oil possessed maximum antifungal activity and the extracts of *Cassia alata* failed to evince the ability to inhibit the growth of fungal iso lates The antifungal activity of plant extracts were compared with the commonly antifungal chemotherapeutic agents