MICROBIAL LOAD IN FROZEN BULL SEMEN AND ANTIBIOGRAM OF THE ISOLATES

BY

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THESIS

Submitted in partial fulfilment of the
requirement for the degree

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
Mannuthy, Thrissur

1996

DECLARATION

I hereby declare that the thesis entitled **"MICROBIAL LOAD IN FROZEN BULL SEMEN AND ANTIBIOGRAM OF THE ISOLATES"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis, entitled **"MICROBIAL LOAD IN FROZEN BULL SEMEN AND ANTIBIOGRAM OF THE ISOLATES"** is a record of research work done independently by **Sri. Roy Mathew**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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ACKNOWLEDGEMENT

I am grateful to my guide Dr. R. Madhusoodanan Pillai, Associate Professor Department of Microbiology and Chairman of the Advisory Committee for his valuable suggestions tolerance and patient correction of several of my short comings as a research student.

I am indebted to Dr. Sulochana, Professor and Head, Department of Microbiology for her inspiring advice encouragement and constructive criticism as member of the Advisory Committee.

I am obliged to Dr. V. Jayaprakasan, Associate Professor and Dr. E. Mathai, Professor, Department of Animal Reproduction for their been interest in the study as members of the Advisory Committee.

The guidance and suggestions extended by Dr. P.C. James, Dr. K.T. Punnoose and Dr. Krishnan Nair, Department of Microbiology are gratefully acknowledged.

I express my sincere thanks to the staff of Dhoni Farm and Mattupetti farm particularly Dr. G. Dinesh, Dr. Kishan, Dr. Gigibhai, Dr. Jose James and Dr. C.P. Gopakumar for their help during the course of this work.

I extend my gratitude to the post graduate students of the Dept. of Microbiology, Dr. A.V. Harikumar, Dr. M. Anilkumar, Dr. S. Manoharan, Dr. N. Venkidabady, Dr. P.S. Jayakumar, Dr. K. Sukumar, Dr. John Hudson and Dr. Sangeetha Vijaysri for the inspiration given in the progress of this work.

ROY MATHEW

Dedicated to my
Beloved Parents

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ABBREVIATIONS EXPANSION

A	-	Ampicillin
Am	-	Amoxycilin
C	-	Chloramphenicol
Cf	-	Ciprofloxacin
Cm	-	Cotrimazine
Cp	-	Cephalexin
Cr	-	Cephaloridine
Cx	-	Cloxacillin
Do	-	Doxycycline
E	-	Erythromycin
Fr	-	Furazolidone
G	-	Gentamicin
Nf	-	Nitrofurantoin
Nx	-	Norfloxacin
O	-	Oxytetracycline
Pb	-	Polymixin B
Pf	-	Pefloxacin
Pg	-	Penicillin G
S	-	Streptomycin
Sz	-	Triple Sulpha

Introduction

INTRODUCTION

Frozen semen is being increasingly used for the improvement of production potential of livestock in this state. The frozen semen for artificial insemination should be of better quality in order to achieve anticipated results.

One of the disadvantages of using frozen semen for artificial insemination is that it can act as a source of infection to a large number of female population if the semen is contaminated with microbial agents. The fluids used for semen extension and the different semen processing procedures involved in long term preservation can very well preserve the different micro-organisms present in extended semen.

Semen consists of spermatozoa suspended in seminal plasma. The seminal plasma is formed from the fluids from testis, epididymis, ampulla, prostate, vesicular and bulbo-urethral glands. A healthy bull ejaculates five to eight millilitres of semen and during its course to the outside, it passes through urethra, which is also the pathway for urine. Semen can be contaminated by micro-organisms reaching it from the testes or any of the accessory organs. It is also exposed to the urethra, preputial cavity and the preputial orifice, all of which contain a large microbial flora. Other than these sources semen gets contaminated by blood or tissue fluid

extravasated into the urogenital system, by microorganisms present in the atmosphere, on the teaser animal, in unsterilized equipment, in the semen extender or from liquid nitrogen or its container (FAO, 1981).

The rich nutritional status and the presence of different cryoprotectant materials in the semen extender used for freezing helps in the perfect preservation of the microbial viability. The bacterial cell because of its diploid nature seems to be more resistant to freezing than the haploid sperm cell.

During the development of the Artificial Insemination industry, there was immense concern about the effect that micro-organisms might have on semen quality and female fertility. The introduction of hygienic procedures and the obtaining of acceptable pregnancy rates served to reduce these fears, but later the realisation that many pathogens could survive both cryopreservation and the addition of antibiotics to semen extenders and thus transmit infectious diseases reawakened interest in this area and led to health regulations being developed for bull studs and the movement of semen.

The processed semen should be free of specific pathogens like *Brucella abortus*, *Campylobactor fetus* and *Trichomonas fetus*. However, there lacks unanimity with respect to permissible limits of non-specific bacterial load in the

insemination dose. The Indian Standards Institution (ISI, 1976) has prescribed a permissible limit of 500 non-pathogenic bacteria per dose of frozen semen used for insemination.

There are a number of research papers on the bacterial load of fresh and extended semen of bulls, but investigation on bacterial and fungal content of frozen semen are comparatively few (Pospelove *et al.*, 1973; Wierzbowski, 1981 and Raghavan *et al.*, 1982).

There is no published reports on the bacterial load of frozen semen of crossbred bulls used in Kerala. Antibiotics like penicillin and streptomycin which are conventionally added to semen extenders are found to be ineffective in checking the bacterial load in frozen semen. This resulted in the reduction in the fertility rate. Previously the farmer had two inseminations free of cost following the first artificial insemination. Now the Government of Kerala has introduced a new system in which the farmer has to pay for every dose of semen. In order to help the farmers, the semen produced for artificial insemination should be supreme in quality.

The present investigation was therefore undertaken with the following objectives.

1. Assessment of bacterial and fungal load of frozen bull semen

2. Isolation and identification of bacteria and fungi present in the frozen bull semen.

3. Study the antibiogram pattern of the isolates so that the study may be helpful to advocate suitable antimicrobial agents as additives in frozen semen to reduce the bacterial load as compared to penicillin and streptomycin.

Review of Literature

REVIEW OF LITERATURE

Total microbial load in bull semen

Rosza (1950) found that the bacterial count of regular collections of semen from eight healthy bulls averaged 80,000 per cubic centimetre which ranged from 250 to 400,000. Marinov *et al.* (1966) examined 480 semen samples from 20 bulls and found that the bacterial count varied from 1.2 to 60 million per millilitre.

The aerobic bacterial flora of ejaculates from 15 bulls was investigated by Meredith (1970). The mean bacterial population per millilitre of ejaculate was 40.4×10^3 for the seven control bulls and 365×10^3 for the eight bulls seen to evert preputial epithelium.

The standard plate count for semen samples from 54 bulls was 57 ± 44 thousands per millilitre (Reddy *et al.*, 1971). They observed that the bacterial count of preputial washings and of semen of bulls with tucked up sheath, was lower than those of bulls with pendulous sheath. As the age of bulls increases, the bacterial count of the preputial washings also increases. They also reported that in young bulls of less than four years of age the bacterial load was 43 ± 24 thousands per millilitre while in older bulls the count rose upto 101 ± 89 thousands per millilitre.

Brown *et al.* (1974) found that the total number of viable microorganisms in semen from 42 bulls used for Artificial Insemination in Newzealand, varied between 20 and 5,34000 per millilitre with a mean of 4630 per millilitre. They also reported that the bacterial population of extended semen stored at 15°C declined to a low level in three days, but the yeast population increased and constituted most of the viable count after two days.

Bacteriological studies were made on 1293 ejaculates from 146 bulls in an Artificial Insemination Centre. Two hundred and eight ejaculates were free of bacteria and the remaining ejaculates contained varying population of bacteria (Balashov *et al.*, 1977).

Kher and Dholakia (1984) reported that the bacterial content of semen varies according to season. The bacterial contamination was high in summer season followed by monsoon and winter. During the summer season the bacterial load per millilitre of fresh semen varied in different breeds of cattle - 11,471 (Gir); 11,920 (Jaffarbadi); 6940 (Jersey) and 6300 (Holstein Friesian). They also reported the influence of age of bull in relation to microbial load in semen. The semen from bulls above six years of age have shown higher bacterial load than younger bulls.

Out of the 7636 samples of semen examined bacteriologically by Stoyanov (1985), no contamination was detected in 42 per cent while contamination was slight in 40 per cent and severe in 18 per cent.

A total of 34 semen samples collected from 22 breeding bulls were subjected to bacteriological examination by Saikia *et al.* (1987). Thirty samples revealed the presence of one or more than one type of bacteria.

Aleem *et al.* (1990) carried out bacteriological examination of semen samples from sixty bulls and found that 27 bulls (45%) produced semen which were contaminated. Seventeen of them had a single species of bacteria and the remaining 10 had mixed flora.

Total microbial load in frozen semen of bulls

Pospelov *et al.* (1973) enumerated the bacteria in frozen semen of bulls and found that the number of bacteria averaged 75,000 per millilitre, ranging from 51,400-2,25000 organisms per millilitre. He also observed that the liquid nitrogen which was used to store semen contained many species of bacteria.

Bacterial load in frozen semen from 1300 ejaculates of 419 bulls was studied by Wierzbowski *et al.* (1973). Twenty nine

per cent had less than 10,000 bacteria per millilitre, 39 per cent contained 10,000 to 100,000 organisms, 26 per cent had 100,000 to 1000,000 and 6 per cent had more than one million organisms.

In another study employing 2856 ejaculates from 461 bulls, an average of 178842 bacteria were isolated per milliliture of frozen semen (Wierzbowski and Szmyd, 1976). There were significant difference between the number of isolates of bacteria in different Artificial Insemination Centres.

Kurudzhiiski (1979) carried out studies on 123 samples of semen which had been stored in deep frozen state in plastic ampuoles, straws or as granules. From these samples of semen 212 strains of bacteria were recovered and identified. The degree of contamination was lower in plastic straws and highest in ampuoles.

From the data collected from 3619 samples of frozen semen Nowakowski and Wierzbowski (1980) observed that the average bacterial contamination was 60,000 bacteria per millilitre

Raghavan *et al.* (1982) reported that all the 34 samples from different ejaculates of five bulls falls within the limits of the international standards of 500 bacteria per dose of semen.

Gangadhar *et al.* (1986) reported that there were significant differences in the total and specific bacterial load per straw of frozen buffalo semen in samples procured from three different centres. The mean values were 110.61 ± 25.01, 2639.84 ± 764.89 and 30.54 ± 6.98 for the three centres.

Nimai Singh *et al.* (1990) found that the mean bacterial in the semen without the addition of antibiotics, reduced from 11.56 ± 1.67 x 10^{10} per millilitre before freezing to 5.84 ± 0.19 x 10^{3} per millilitre immediately after freezing and to 2.50 ± 0.16 x 10^{3} per millilitre, ninety days post freezing.

Types of bacteria and fungi in semen

Specific pathogenic bacteria

The transmission of brucellosis by artificial insemination was thoroughly described by Bandixen and Blom (1946). One bull transmitted brucellosis to 71 per cent of females inseminated.

A bull in service from 1957 was found to have generalised tuberculosis including pelvic lesions, on slaughter after six years of service. Semen from the bull had been used to inseminate more than 1000 cows, and more than 100 were found to have tuberculosis of internal organs, on slaughter (Raumy, 1966).

An experimentally added *Mycoplasma agalactia* var *bovis*, a natural pathogen of the female genital tract, survived the addition of penicillin G and dihydrostreptomycin and was found to be alive in the semen after 18 months of storage at -196°C (Hirth, 1967).

Larzen and Kopecky (1970) reported that out of the seven bulls which had clinical signs of paratuberculosis, *Mycobacterium paratuberculosis* was recovered from the semen of one of them.

Rodina (1970) reported that *Leptospira pomona*, *L. hebdomadis* and *L. canicola* when added to semen samples remained pathogenic upto three months storage at 3°C to 23°C. The organism survived and remained fully pathogenic for atleast three years when the infected semen was frozen and stored at -196°C.

Belik (1975) carried out studies on semen samples and preputial mucus from breeding bulls. Out of a total of 282 samples of semen and preputial mucus, 104 isolates of *Campylobacter fetus* were recovered.

Erno (1975) reported that 7.8 per cent of the semen samples from Danish Artificial Insemination bulls contained mycoplasmas. The majority of the strains isolated belonged to

Mycoplasma bovigenitalium (85%) and the remaining strains were Acholeplasma (13%) and the rest were *Mycoplasma bovirhinis*.

Lein and Nielsen (1975) studied urogenital mycoplasmosis in 17 bulls, using *Mycoplasma bovigenitalium* and *M. agalactia* subsp *bovis*. Following infection, the organism were continuously secreted into semen or seminal fluids.

Juramanova *et al*. (1977) isolated 56 Mycoplasma strains from 202 semen samples from insemination centres in eastern Bohemia during an year period. The species isolated were *Mycoplasma bovigenitalium*, *M. bovirhinis*, *M. agalactia* and *Ureaplasma* sp. On the basis of comparison of the motility, concentration of ejaculates and the presence of the Mycoplasma, a relationship was shown between inferior quality of the ejaculates and the concentration of Mycoplasma (Juramanova and Sterbanova, 1977).

Hodges and Holland (1980) reported the recovery of Ureaplasma from 24 out of 50 semen samples, 19 out of 25 preputial scrapings and 10 out of 25 preputial washings from one hundred clinically healthy bulls.

Microbiological examination of 780 semen samples taken from breeding bulls showed 52 (7.4%) infected with *Haemophilus somnus* (Jansen *et al*., 1981).

Larsen *et al.* (1981) recovered *Mycobacterium paratuberculosis* from all the 26 faecal samples during a period of 21 months from a bull infected with the organism. Only eight of the 31 semen samples tested contained the organism. Routine faecal cultures at bull studs will reveal infected bulls before they become genitally infected and shed *M. paratuberculosis* in semen.

Mycoplasma bovigenitalium was isolated from the vesicular fluid of four out of 20 bulls (Panangala, 1981). The serological tests showed higher antibody titres in vesicular fluid than in plasma.

Pathogenecity studies of *Mycoplasma bovis* revealed that direct intravesicular infection of *M. bovis* resulted in seminal vesiculitis with persistent shedding of Mycoplasma in the vesicular gland secretions for a period upto 8.5 months (Lafaunce and McEntee, 1982).

Campylobacter fetus subsp. *venerealis* is found in the preputial cavity and the distal portion of urethra. Transmission of the infection occurs during coitus or by artificial insemination procedures, as the organism is found in bovine semen (Garcia *et al.*, 1983).

Ungureanu *et al.* (1984) recovered 24 strains of Mycoplasma and 49 strains of Ureaplasma from 310 samples of deep frozen

semen. In growth inhibition test against 11 monospecific antimycoplasma sera, 20 of the 24 Mycoplasma strains were identified as *M. bovigenitalium* (13), *M. bovis* (1), *M. canedense* (1), *M. californicum* (2), *M. bovirhinis* (1) and *Acholeplasma laidlawii* (2). Four isolates were not identified (Ungureanu *et al.*, 1985).

Campylobacter fetus was not identified from the prepuce or from semen samples when 90 bulls in the Ontario Artificial Insemination units were screened by penile glove swab technique (Finley *et al.*, 1985).

In a survey on 45 breeding bulls, Mycoplasma or Ureaplasma were isolated from 43 bulls. The organism were found in the prepuce of 41 bulls, in fresh semen of 24, in nine samples of processed semen, in the urethral orifice of 33, in the seminal vesicles of nine and in the testicles of one (Fish *et al.*, 1985).

Fejes (1986) isolated Mycoplasma repeatedly from 120 breeding bulls in an AI station. *Mycoplasma bovigenitalium* was isolated in 78.8 per cent, *Acholeplasma laidlawii* in 9.1 per cent, *M. bovirhinis* in 6.1 per cent and other species in 6.1 per cent from positive bulls. The occurrence of pathological changes in 33 bulls carrying Mycoplasma was about three times higher than that of bulls with no Mycoplasmas. The report

also points out that the semen quality of Mycoplasma carriers was usually inferior.

Garcia *et al.* (1986) reported that *Mycoplasma bovis* was not detected in approximately 2950 unprocessed frozen semen samples from nine Canadian Artificial Insemination centres during a five year study.

Ball *et al.* (1987) observed in their screening studies on 332 fresh and 137 processed bovine semen samples and 25 preputial washings, Mycoplasmas and/or Ureaplasmas in 46 per cent, 31 per cent and 80 per cent of the samples respectively. Atleast three collections per bull were necessary to determine whether infection was present.

Machado and Atalia (1987) isolated *Mycoplasma bovis* and Mycoplasma serogroup 7 of Leach (Pg 50) from bovine semen. The authors studied the pathogenic role of these organisms and stressed the recommendations of the International Organisation for Mycoplasmology (1980-82) on dangers of dissemination of Mycoplasma through the exportation of contaminated semen.

Two hundred and thirty four Mycoplasma strains were isolated from 380 semen samples collected from six AI centres. They were identified as *Mycoplasma bovigenitalium* (75%), *M. bovis* (7.8%), *M. bovirhinis* (1.9%), *M. arginini* (1.3%),

Acholeplasma laidlawii (8.5%) and *A. modium* (0.7%) (Marciczewska, 1987).

Nielsen *et al.* (1987) developed a capture immuno assay for the detection of *Mycoplasma bovis* antigens in bull semen or preputial washings.

Breard and Poumarat (1988) in their routine examination of the semen of a breeding bull isolated *Mycoplasma bovigenitalium* and *Mycoplasma capricolum.*

Richter *et al.* (1989) after the bacteriological examination of several frozen semen samples, concluded that reliable information on contamination with Mycoplasma were obtainable by testing three pellets from a given batch of frozen semen.

The examination of frozen semen samples from 93 AI bulls showed that two were positive for Mycoplasma and those two had oligospermia (Trifunovic, 1989).

Rosario-Gonsalves (1990) recorded the isolation of Mycoplasma from the semen of 18 out of 22 bulls in an AI centre. Mycoplasma elimination was intermittent, bulls being positive for between one and three out of six semen samples collected at fortnightly interval.

Pal (1993) during a five year surveillance programme on semen samples of 287 breeding bulls isolated Mycoplasma/Acholeplasma from 113 (39.3%) animals. The prevalence in bulls of Haryana, Jersey, Holstein Friesian crossbreds and Jersey crossbreds was found to be 34, 41, 45, 37 and 14 per cent respectively.

Non-specific bacteria in bovine semen

Gamick (1950) reported the presence of *Pseudomonas pyocyanea* in the semen of nine out of 11 bulls at an insemination centre. Blom and Romer (1961) isolated 69 strains of streptococci from 90 out of 2292 semen samples from 2018 healthy bulls.

Semen samples from 1500 bulls in Danish AI stations were screened for microbial agents. *Escherichia coli* was isolated from 637 (19%) of 3272 samples (Blom and Dam, 1966). Marinov *et al.* (1966) reported the presence of *Proteus vulgaris*, *Pseudomonas aeroginosa*, *Escherichia coli* and *Bacillus megatherium* in samples collected from 20 breeding bulls.

Reddy *et al.* (1971) observed the presence of *Bacillus* sp. *Staphylococcus epidermidis*, *Staphylococcus pyogenes*, *Proteus vulgaris*, *Pseudomonas pyocyanea*, *Alkaligenes faecalis* and coliforms in semen samples.

Pospelov *et al.* (1973) reported that semen samples stored in granules in liquid nitrogen always contained *Staphylococcus aureus*, *Staphlococcus albus*, *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium*. Liquid nitrogen used for storage of semen was traced as the source of Staphylococcus, Sarcina, Proteus and *E. coli*.

Bacteria belonging to the genera *Bacillus*, *Corynebacterium*, *Micrococcus*, *Aerobacter*, *Alkaligenes*, *Escherichia*, *Flavobacterium*, *Herellea*, *Nocardia*, *Proteus*, *Sarcina* and *Staphylococcus* were obtained from 42 bulls used in an artificial breeding station at Newzealand (Brown *et al.*, 1979).

Corynebacterium renale belonging to type II and type III were isolated from 60 (52%) of the 116 semen samples (Hiramune *et al.*, 1975). Kirchhelle (1975) subjected one hundred Micrococcaceae strains isolated from bull semen, to various cultural and biochemical differentiation methods and characterized the isolates as either belong to genus Staphylococcus or to genus Micrococcus.

Pseudomonas aeroginosa was detected as a common contaminant of bull semen by several workers (Mikhailov *et al.*, 1975; Palk *et al.*, 1976; Nowakowski *et al.*, 1980; Raghavan *et al.*, 1982).

Presence of *Staphlococcus* sp. in bull semen appeared in several reports (Meyer *et al.*, 1980; Nowakowski *et al.*, 1980; Saikia *et al.*, 1987; Danowski, 1989).

Naidu *et al.* (1981) reported the isolation of *Pseudomonas* spp., *Corynebacterium* spp. *Micrococcus* spp., *Staphylococcus* spp. *Proteus* spp. *E. coli* and *Alkaligenes* spp. from six Murrah buffalo bulls and six Jersey bulls.

Rahman *et al.* (1983) reported the most important contaminants in semen samples as *Staphylococcus aureus*, Streptococcus, *Escherichia coli*, *Klebsiella aeroginosa* and *Pseudomonas aeroginosa*.

Gangadhar *et al.* (1986) observed the presence of *Enterobacter liquifascians* and *Pseudomonas aeroginosa* in buffalo bull semen. Hussain *et al.* (1990) reported the prevalence of *E. coli* 21.1 per cent; *Bacillus subtilis* 17.9 per cent; *Proteus vulgaris* 16.3 per cent; *Micrococcus* sp. 11.9 per cent; *Pseudomonas aeroginosa* 9.2 per cent; *Corynebacterium pyogenes* 5.5 per cent; *Streptococcus hemolytica* 4.3 per cent; *staphylococcus aureus* 3.8 per cent and unidentified Gram negative bacilli 2.1 per cent in bull semen.

Eaglesome *et al.* (1992) reviewed the potential pathogens and semen contaminants commonly encountered during the bacteriological examination of bull semen.

Gradil *et al.* (1994) explored the potential use of polymerase chain reaction for the detection of *E. coli* present in the semen with the help of verotoxin 2 (VT 2) gene.

Yeast and moulds in semen

Horter (1962) examined preputial samples from 293 bulls for fungi of which 38 (13%) yielded fungi. The isolates include Aspergillus, Penicillium, Mucor, Rhizopus, Graphium, Cephalosporium, Phoma, Oosporium, *Fusarium roseum* and Scopularopsis.

Hajsig *et al.* (1968) experimentally infected 43 mice with the yeast *Hansenula anomala* isolated from the semen of breeding bulls. Nineteen of them died within 21 days of inoculation indicating that the yeast present in the semen were pathogenic.

Zvareva and Repco (1968) reported the presence of fungi belonging to the genus Aspergillus, Lichthemia, Cephalosporium, Candida, Actinomyces, Penicillium, Helminthosporium, Alternaria, Mucor, Trichophyton and Fusarium from undiluted and diluted semen samples. Sixty four per cent of the bull semen were contaminated with fungi.

Fungi were cultured from the preputial washings of 103 (36%) of 285 bulls in spring and 60 (22%) of 302 in autumn from

12 AI centres. The centres with the highest contamination had the lowest fertility (Rob and Toman, 1970).

Brown *et al.* (1974) reported the isolation of the yeast *Rhodotorulla rubra* and *Candida guilliermondi* from the semen of bulls in Newzealand.

Richter (1975) observed a correlation between the presence of yeast in the food and in the semen. Their presence was also correlated with lowered fertility. He hypothecated that the moulds and yeasts were absorbed from the food and excreted through the semen.

Nine species of mycelial yeasts were obtained from 129 samples of semen from 62 bulls. *Candida parapsilosis* and *Candida tropicalis* were the most frequently isolated organisms (Richard *et al.*, 1976).

Stoyanov (1985) reported that out of the 7636 semen samples examined, fungi were detected only in 0.01 per cent of the samples.

Gangadhar *et al.* (1986) obtained 39 isolates of unidentified yeasts together with 33 isolates of penicillium and 19 of *Aspergillus niger* from 221 samples of 46 bulls.

Candida tropicalis, *Candida guilliermondii* and *Rhodotorulla* sp. were isolated from semen samples of nine Holstein Friesian bulls used for AI (Natalia, 1987).

Influence of the microbial load on the quality of semen

Bush *et al.* (1950) reported a drop in conception rate, as the number of bacteria in semen increased following 11,912 first inseminations. The type of bacteria predominantly in semen was related to fertility. When cows were inseminated with the bull semen from which *Pseudomonas pyocyanea* was isolated, there were disturbances in the oestrous cycle.

Helt (1961) observed a reduction in fertility of semen of three bulls from which *Corynebacterium pyogenes* was isolated. Two of them had the infection localised in the testis and the third bull had the infection in the seminal vesicles.

The lipopolysacharides of *Vibrio fetus*, *Pasteuralla multocida* and *Serratia marcescens* upon exposure for three hours killed ram spermatozoa. Lipopolysaccharides of *Serratia marcescens* was the most toxic (Dennis, 1962).

Romaniuk (1965) demonstrated that conception rates were 81 per cent for semen containing bacteria upto 300,000 per millilitre and 73.8 per cent for semen containing over 500,000

per millilitre. Counts of 300,000 or more were regarded as adversely affecting fertility.

Getty and Ellis (1967) described the development of metritis, cervicitis and vaginitis in heifers inseminated with semen containing *Pseudomonas aeroginosa*.

Corynebacterium renale type III recovered from semen samples of healthy bulls, produced fever and hematuria with cystitis when experimentally inoculated into the urinary bladder of a healthy cow (Hiramune *et al.*, 1975).

Lein and Nielsen (1975) reported the development of sperm abnormalities and oospermia following inoculation of *Mycoplasma bovigenitalium* or *Mycoplasma agalactia* subsp. *bovis* into the seminal vesicles.

Juramona and Sterbova (1977) demonstrated that less than 70 per cent motile spermatozoa were found in 12 out of the 44 samples infected with *Mycoplasma* spp., compared with two of 31 Mycoplasma free samples.

The extracellular proteins of *Staphylococcus aureus* damaged spermatozoa as evidenced by loss of motility and loss of acrosomal cap (Meyer *et al.*, 1980).

Nowakowski *et al.* (1980) reported that of the 101 strains of *Pseudomonas aeroginosa* isolated from the semen of bulls,

40 per cent killed mice within 24 hours of intraperitoneal injection, and a further 14 per cent died on the second day.

Panangala (1981) observed that vesicular fluid of four of the 20 bulls examined contained *Mycoplasma bovigenitalium*. Out of this, 33 per cent of the semen samples had spermatozoa motility of 20 per cent or less. The organism can be transmitted via the respiratory route, leading to epididymitis and vesiculitis.

Boryczko (1982) demonstrated that when bull semen was incubated with *Pseudomonas aeroginosa*, *Staphylococcus aureus* and *E. coli* cultures at 37°C for 60 minutes, at a concentration of 30 million organisms per millilitre, a considerable reduction of the percentage of live spermatozoa occurred. The tested microorganisms reduced the oxygen uptake of spermatozoa, and there was also an increase in aspartate amino-transferase activity in the semen samples.

Hare (1985) reviewed the current knowledge regarding the transmission of infectious diseases by semen and its control by accurate diagnostic techniques.

Six hundred and seventy one heifers were inseminated with frozen semen containing Mycoplasmas and the results were compared with 499 heifers inseminated by uncontaminated semen. The presence of Mycoplasma had an adverse effect on conception

rate and on the number of doses of semen needed for conception (Kissi *et al.*, 1985). Fifteen of the 33 bulls from which Mycoplasmas were repeatedly isolated, had pathological changes in their genital organs during autopsy (Fejes, 1986). The semen quality of Mycoplasma carriers was also inferior.

Aleem *et al.* (1990) observed that all the strains of *Staphylococcus aureus* isolated from buffalo semen were hemolytic. Eight of the sixteen *Escherichia coli* and all the strains of Pseudomonas, *Proteus mirabilis* and *Streptococcus pyogenus* were lethal to mice after intraperitoneal injection.

Insemination of female rabbit with semen artificially contaminated with *Pseudomonas aeroginosa*, did not impair embryonic survival (Radchinkov *et al.*, 1990).

Rosario-Gonsalves (1990) reported that no significant difference in motility and concentration of spermatozoa were found between semen samples positive and negative for Mycoplasma. Field strains of Mycoplasma were shown to have sperm agglutinating properties.

Sobiech *et al.* (1991) reported that in young bulls reacting serologically positive to *Chlamydia psittaci* antigen, a decrease in volume of ejaculate, concentration of spermatozoa, number of spermatozoa showing normal movements was observed. An increase in abnormal spermatozoa was also noted.

In studies of 58 bulls positive for *Pseudomonas aeroginosa* in semen samples, the insemination index did not differ significantly when compared to the index of 57 negative bulls (Radchenkov *et al.*, 1993). They also observed that *P. aeroginosa* had no influence on survival of spermatozoa and fertility in examined animals.

Standards of microbial load in frozen semen

Ostaszko (1976) in the studies to standardize the number of saprophytic microorganisms per millilitre of frozen semen with the conception rate, recorded that in order to obtain a conception rate of 66 per cent the microbial load per millilitre of frozen semen must be limited to 500 microorganisms.

The Indian Standards Institution (ISI, 1976) recommended that the frozen semen should be free from pathogenic bacteria. In addition, the number of saprophytic bacteria per dose of frozen bull semen should be kept below 500. In order to keep up the quality of semen ISI advocated that all the frozen bull semen producers has to follow this specification standards.

Willis (1978) also recommended that the maximum number of bacterial organisms present in frozen semen should be limited to 500 per dose.

The limit of 500 organisms per millilitre of frozen semen was recommended by Ostaszko (1976) to the international organisation of biological standards. According to the reported documents of Wierzbowski (1981), the recommendation was not accepted.

Eventhough the limit of 500 organisms per millilitre of frozen semen was not accepted, the limit of 500 organisms per dose of frozen semen was accepted by the international organisation of biological standards (Raghavan *et al.*, 1982).

Effect of antibiotics on the viability and fertility of semen

Rosza (1950) reported that penicillin at a concentration of 30-50 units and streptomycin at a concentration of 0.02 to 0.04 mg per cubic centimetre of egg yolk citrate diluent had an effective bactericidal action against Gram positive and Gram negative bacteria present in semen. Larger doses of the antibiotics were found to be harmful to sperm motility and metabolism.

Lenz (1956) however demonstrated that penicillin, streptomycin combination and penicillin, streptomycin and polymixin combination of antibiotics were not effective when added to semen samples diluted with egg yolk citrate and artificially infected with four strains each of *Corynebacterium*

pyogenes, *Pseudomonas pyocyanea*, *Brucella abortus* and *Vibrio fetus*.

Diliello (1957) reported that aureomycin and terramycin were highly toxic to spermatozoa. Two hundred and fifty micrograms of streptomycin per millilitre was the best single agent found to be effective against three strains of *Vibrio fetus*. Gamcik (1959) observed that a mixture of streptomycin, penicillin and polymixin when added to semen made it practically free of *Pseudomonas pyocyanea*.

The usual addition of penicillin-G and dihydro-streptomycin failed to eliminate *Mycoplasma agalactia* var. *bovis* even after storage under liquid nitrogen at -196°C (Hirth *et al.*, 1967).

Larsen and Kopecky (1970) reported that *Mycobacterium paratuberculosis* can survive procedures similar to the antibiotic treatment and freezing, that are used to process semen commercially.

Brown *et al.* (1974) after testing 37 strains of bacteria against 13 antibiotics, found that the predominant microbial flora was sensitive to a wide range of antibiotics. Pseudomonads, Coliforms and yeasts were the most resistant organisms. Nystatin added to the extender controlled yeast growth. Palli *et al.* (1975) reported that the pseudomonas

obtained from bull semen were most sensitive to neomycin, polymixin, olemorphocycline and were not very sensitive to penicillins and streptomycins.

Minocin with lincospectin eliminated Ureaplasma, *Acoleplasma laidlawii*, *Mycoplasma bovigenitalium*, *M. bovis*, *M. canadense* and *M. arginini* from artificially infected semen. Stabilisation times of 15 minutes at 35°C and three hours at 4°C are important considerations to maximise antibiotic activity (Truscott and Arbeo, 1977).

Froster (1979) reported that *Campylobacter fetus* isolated from semen and abortion cases were resistant to neomycin, kanamycin and lincomycin in Tris diluent at 30°C. Eventhough gentamicin, streptomycin and spectinomycin were only partially effective, their addition to semen diluent can be used as a prophylactic measure.

Kuradshiiski (1980) reported that all the 66 isolates from bull semen were resistant to penicillin, erythromycin, tetracycline, novobiocin, neomycin, kanamycin, chloramphenicol, streptomycin, olendomycin, spectinomycin, furazolidone sulphoxine-trimethoprim, tylosin, oxacillin, and sulphathiazole. But gentamicin, carbenicillin and rifampicin inhibited growth at minimum concentration of 1.5 to 50 ug per millilitre.

Boryczko *et al.* (1981) confirmed that if the concentration of penicillin and streptomycin exceeds 500 units and one milligram per millilitre respectively, the proportion of dead spermatozoa was increased.

Studies on 66 strains of *Pseudomonas aeroginosa* isolated from deep frozen bull semen confirmed that they were removed by a combination of carbenicillin, 64 microgram per millilitre plus gentamicin 3 microgram per milliltire. Satisfactory results were also given by carbenicillin plus kanamycin each at 64 micrograms per millilitre (Kurudshiiski, 1981).

Naidu (1981) observed that the bacterial strains of *Corynebacterium equi*, *Micrococcus* spp obtained from semen were resistant to streptomycin. *Corynebacterium renale C. xerosis* and *Micrococcus* spp. were sensitive to penicillin. *Pseudomonas* spp. were resistant to tetracyclines ampicillin, polymixin, colistin and sulphamethazole. All the isolates were sensitive to gentamicin.

Rahman *et al.* (1983) reported that gentamicin, kanamycin and neomycin were the most effective drug against organisms present in semen. Penicillin and streptomycin were the least effective.

Holzman *et al.* (1984) reported that Tiamulin, a new antibiotic at a concentration of upto 70 milligram per 100

millilitre of semen diluent was safe and nontoxic for spermatozoa. Tiamulin effectively eliminated Mycoplasma from semen.

Foote and Ahmad (1986) studied the safe level of six antibiotics to be added to extended semen. Percentage of motile spermatozoa in milk diluent declined when concentration of dicloxacillin exceeded 200 microgram per millilitre or when gentamicin exceeded 500 ug per millilitre. Minocin was toxic at all levels tested in egg yolk and was nontoxic to sperm in milk extender at concentrations below 100 ug per millilitre. Cephapyrin and ceforanide were innocuous at all concentrations tested (200-2000 ug per millilitre). Fertility with cephapyrin 500 ug per millilitre added to an extender resulted in a 56 day non-return rate of 75.5 versus 72.1 percentage from the control.

Truscott (1986) demonstrated that flourafamide at 1500 ug per millilitre did not reduce ureaplasma in naturally infected semen diluted in phosphate buffer at pH 6.5 and held at 35°C for 20 minutes cooled over 2.5 hours and held at 5°C for one hour.

Addition of lincomycin and spectinomycin to semen extenders eliminated Mycoplasmas and reduced the number of Ureaplasma (Ball *et al.*, 1987).

Natalia (1987) reported that mycostain (nystatin) at 50-100 units per millilitre could be added to diluted semen without affecting the viability or motility of bovine spermatozoa.

Saikia *et al.* (1987) observed that the microorganisms present in semen were highly sensitive to gentamicin (100%) followed by kanamycin (94.59%), neomycin (89.18%) and ampicillin (83.78%). Less effective drugs were bacitrain (0%), polymixin-B (27%), penicillin (10.81%) and streptomycin (45.94%).

Shin *et al.* (1988) reported a new combination of antibiotics - gentamicin (500 ug/ml) tylosin (100 ug/ml) and linco-spectin (300/600 ug/ml) was more effective for the control of *C. fetus* subsp. *venerealis* and *Haemophilus somnus* than the standard combination of penicillin, dihydro-streptomycin and polymixin-B sulphate.

Lorton *et al.* (1988) also demonstrated that the above combination is not detrimental to seminal quality and confirmed in parallel studies that it was the most efficient combination in controlling micro-organisms present in bovine semen. This combination of antibiotics were used to study the non-return rates in females (Lorten *et al.*, 1988b). Results indicated no significant effect on seminal quality as measured by field fertility as compared to control in which the antibiotic

combination of penicillin, dihydrostreptomycin polymixin B sulphate with/without linco-spectin (500 units/ml, 2000 ug/ml, 1000 units/ml and 300-600 ug/ml respectively) were used.

Ahmed (1989) reported that there was no difference in sperm motility between treatments when samples were treated either with penicillin 800 IU and streptomycin 1000 ug/ml or gentamicin 500 ug/ml. The samples with gentamicin contained no bacteria. However Hardjoutomo (1990) reported that streptomycin and penicillin were very much effective against semen contaminants at the Lembang Artificial Insemination Centre.

Antibiotic sensitivity tests of the isolates from semen samples revealed that chloramphenicol and gentamicin were most effective against Gram positive and Gram negative organisms respectively. Assessment of the microbial load at different hours of preservation (both refrigeration and liquid nitrogen) was carried out and was found to be significantly low throughout when compared with controls in which no antibiotics were added (Nimai Singh, 1990).

Incubation of semen samples with antibiotics such as penicillin (500 IU), streptomycin (500 ug), lincomycin (160 ug) and spectinomycin (300 ug) per millilitre for 5, 10, 20 or 40 minutes before freezing reduced the number of *Campylobacter fetus* to non-detectable levels in 38 per cent, 69 per cent,

88 per cent and 100 per cent of samples respectively. There was no significant reduction in sperm motility (Shisong *et al.*, 1990).

Kupferschmied *et al.* (1991) inseminated 2108 heifers and 8930 cows with semen diluted in Tris-egg yolk extender containing penicillin and streptomycin or gentamicin, tylosin, lincomycin, and spectinomycin. The 75 day non-return rate to first service was 72.3 and 62.36 per cent respectively.

Wayda (1991) suggested a combination of gentamicin and penicillin for the preparation of diluent and cryopreservation since, out of the 137 bacterial strains isolated from 353 frozen semen samples, 124 were resistant to penicillin and 89 to streptomycin. All the strains were sensitive to gentamicin.

Eaglesome *et al.* (1994) studied the effect of photosensitive agents for disinfection of bovine semen. Haematoporphyrins, haematoporphyrin derivative and thiopyronin were effective against *Mycoplasma bovigenitalium*, *M. canadense* and *Ureaplasma diversum*. Thiopyronin was effective against *Leptospira interrogans* serovar *pomona*. The photosensitive agents were not effective against *L. interrogans* serovar *hardjo*, *Mycoplasma bovis* or *Campylobacter fetus* subsp. *venerealis*.

Materials and Methods

MATERIALS AND METHODS

Bacterial and fungal load in frozen semen samples of crossbred bulls

Procurement

For conducting this work on the bacterial and fungal load of semen samples of crossbred bulls, 132 frozen semen straws of 0.25 ml capacity and 16 straws of 0.5 ml capacity were procured from three frozen semen production centres in Kerala, viz., Mannuthy, Dhoni and Mattupetti. These production centres were designated as centres A, B and C respectively throughout this study. In centre A, frozen semen was produced from a few bulls only for experimental and demonstration purpose. Centre B and C come under the Kerala Livestock Development Board Limited and are concerned with large scale production of frozen semen used for Artificial Insemination in this state. The frozen semen straws were picked at random from the bulk storage containers from these centres and transported to the laboratory at the College of Veterinary and Animal Sciences in a two litre liquid nitrogen container.

Centre-wise procurement of straws were as follows:

Centre A - 16 straws of frozen semen from four collections of two bulls

Centre B - 66 straws of frozen semen from 11 collections of 11 bulls

Centre C - 66 straws of frozen semen from 15 collections of 11 bulls

Thawing and dilution of samples

Thawing and dilution of the samples were done according to the recommendations given by Office of the Internationale Epizooticae (OIE, Code 1992). The samples of semen kept in liquid nitrogen were thawed in sterile distilled water at 37°C for two minutes. Dilution media composed of buffered peptone water (Appendix A-10) was first poured into plastic screw capped bottles. Nine millilitres of dilution media was taken in each bottle.

After thawing, the straws were dried and disinfected using 70 per cent ethyl alcohol. Six 0.25 ml straws or four 0.5 ml straws from a single ejaculate were used for each study. One ml of thawn semen was diluted in the first bottle containing nine millilitres of dilution media. This was thoroughly mixed and one millilitre from this was transferred to the second bottle. Thorough mixing was done again and one millilitre was transferred into the third bottle. The exact dilution rate was determined based on preliminary study of bacterial load of three to four straws from each centre.

Estimation of total and specific bacterial load

The total and specific bacterial count were estimated by pour plate technique (Cruickshank *et al.*, 1975).

Five hundred microlitres each of the serially diluted thawn semen samples were transferred into sterile duplicate petriplates using micropipette fitted with sterile disposable plastic tips. The following media previously sterilised were held at 50°C in a water bath. These were poured into each dilutions of the semen, mixed well and held horizontally to solidify.

1.	Tryptic soy agar containing 5% defibrinated bovine blood (Blood Agar)	-	For total bacterial count (OIE Code 1992, Bovine semen)
2.	Eosin methylene blue agar	-	For total coliforms count
3.	Pseudomonas isolation agar		
	(i) for pyocyanin		
	(ii) for flourescin	-	For Pseudomonas count
4.	McConkeys agar	-	For total lactose and non-lactose fermenters count
5.	Mannitol salt agar	-	For total staphylococci/ micrococci count
6.	Mueller Hinton agar	-	For total gram positive and gram negative count
7.	PPLO agar	-	For total mycoplasma count
8.	Potato dextrose agar	-	For total yeast and mould count

The details regarding the composition of the media are given in the Appendix A. The plates were incubated at 37°C for 72 hours. Petriplates containing different media without diluted semen were also incubated along with the other plates for checking the sterility of the media. The plates containing potato dextrose agar was incubated at 37°C and at room temperature for seven days.

At the end of incubation the number of colonies were counted in an illuminated Quebec colony counter. The mean number of colonies counted in the plates for the highest dilution of each of the ample was taken as the total count for that particular medium. The total and specific bacterial load were determined by multiplying the mean colony count with the dilution factor. The bacterial load was estimated per straw (per insemination dose) irrespective of the volume of the straw.

Identification of the bacterial isolates

The total number of colonies in all the media were counted and representative colonies were picked and subcultured into tryptose soya agar. Isolates were identified upto the species level. Each representative colonies was studied with respect to its colony character, Gram's staining reaction, Acid fast staining, spore staining and motility (Cruickshank *et al.*, 1975).

Other than the above mentioned characters the following tests were carried out to elucidate the identity of the isolates and all the tests were carried out as per the standard procedures described by Cruickshank *et al.* (1975).

Tests for metabolism of carbohydrate and related compounds

Carbohydrate fermentation test

Twelve sugars were used for the test. The sugar under test was incorporated at 1 per cent level into peptone water medium containing phenol red indicator. The organism under test were inoculated into the fermentation tube and incubated at 37°C for 48 hours. Negative cultures were incubated for maximum 40 days. The sugars used for the fermentation tests were Xylose, Glucose, Fructose, mannose, Galactose, Sucrose, Maltose, Lactose, Trchalose, Cellobiose, Dulcitol, Inositol and Salicin.

Methyl red test

The test was done on a 48 hour culture grown in glucose phosphate peptone water Negative cultures were incubated for upto five days at 37°C Addition of about five drops of the methyl red reagent at the end of incubation gave a bright red colour in positive cases and an yellow colour in negative cases.

Voges-Proskauer test

The test was done on 48 hour cultures of the organisms at 37°C in glucose phosphate peptone water. The test was done according to Barritt's method (Cruickshank *et al.*, 1975).

Citrate utilization test

The test was done by streaking the cultures on Simmon's citrate agar (Appendix B-8). In positive case, there was blue colour and growth on the streak whereas the original green colour was retained in negative cases.

Gelatin liquifaction

Nutrient gelatin was prepared in tubes (Cruickshank *et al.*, 1975) and a stab culture of the organisms was made using inoculum from an agar slope culture. The tube was incubated at 37°C. Liquifaction of gelatin was tested at intervals by removing the nutrient gelatin cultures from the incubator and holding them at 4°C for 30 minutes before reading the results. Negative tests were observed for 30 days.

Hydrogen sulphide production test

Triple sugar iron agar (Appendix B-10) was used for the production of hydrogen sulphide. In positive cases there was

blackening of the medium. The test was done as described by Sonnenwirth (1980).

Indole test

The test was done on 48 hours liquid culture grown in two per cent peptone water containing 05 per cent sodium chloride at 37°C. Addition of 0.5 ml of Kovac's reagent (Appendix B-4) resulted in development of red colour in indole positive cases.

Amino acid decarboxylase test

Two amino acids, ornithine and lysine were used in this study. The media were prepared as described in the appendix B-1 and the isolates under study were inoculated. This was incubated at 37°C and read daily for four days. Decarboxylation of the aminoacids was indicated by the development of a violet colour in the medium.

Malonate utilization and phenyl alanine deaminase test

The test medium was prepared as described in appendix B-5. After inoculation, the medium was incubated at 37°C, overnight. Malonate utilisation was denoted by the development of a blue colour. After recording this results the medium was acidified with a few drops of 0.1 N HCl. For testing the phenyl alanine deaminase activity, a four drop of 10 per cent aqueous solution

of ferric chloride was added. A dark green colour developed in positive cases.

Catalase test

A 24 hour culture of the organism was picked with a glass rod and inserted into 3% W/V hydrogen peroxide solution. The presence of the enzyme, catalase, was denoted by the formation of air bubbles from the culture material.

Oxidase test

Filter paper strips to which the reagent (tetramethyl-p-phenylene-diamine dihydrochloride) was absorbed were utilised for this purpose. The bacterial colony was taken with the tip of a sterile glass rod and smeared on to the filter paper strip. Presence of a blue colouration at the site of smearing within five to ten seconds was taken as positive.

Urease test

The slope of Christensen's urea medium (Appendix B-2) was heavily inoculated with a 24 hour culture of the bacterial isolate and incubated at 37°C for upto four days. Urease positive organisms changed the colour of the medium to purple pink.

Nitrate reduction test

Nitrate broth and the reagents were prepared as described in (Appendix B-6). The medium was inoculated with the culture and incubated for 96 h. Equal volumes of the two reagents A and B were mixed and 0.1 ml of this was added to the test culture. Development of red colour following the addition of above reagents indicated that the isolate could reduce nitrate to nitrite.

Haemolysin production

Haemolysin production of the bacterial isolates were studied by inoculation of the organism into 5 per cent bovine blood agar and incubating at 37°C.

Coagulase test

0.1 ml of an 18 hr broth culture of the strain was inoculated into 1 ml of a 1 in 10 dilution of rabbit plasma in normal saline. This was incubated in a waterbath at 37°C and examined for coagulation at 1, 3 and 6 hours. Formation of a gel indicated a positive reaction.

Identification of the yeasts and moulds

The isolates of yeast obtained from the semen samples were identified based on their microscopic morphology, growth on

corn-meal agar containing Tween 80, germ tube test, urease test and sugar fermentation test.

Cornmeal agar cut-streak culture

Plates of cornmeal agar (Appendix B-3) 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 cover slip was placed over this and pressed down. It was examined, both under low power and high power, for sporulation of the yeast.

Germ tube test

The yeast colony was taken from the culture plate and emulsified in 0.5 ml of sterile bovine serum. The tubes were incubated for two and a half hours in a 36°C waterbath. 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

Christensen's urease agar (Appendix B-2) slants were prepared and inoculated with the yeasts. This was incubated at 30°C for a week. The test was recorded as positive when the colour of the medium changed to red-violet.

Sugar fermentation test

Three day old yeast culture was emulsified in sterile distilled water to get a density equal to tube # 1 of Mcfarland's standard. 0.2 ml of this suspension was added aseptically into the fermentation tubes (Appendix B-9) containing Durham's vial. One tube was inoculated without sugars to serve as a control. All the tubes were incubated at 30°C for 7 days. The change in pH and the presence of gas in Durham's tube indicated positive fermentation to the sugar in tubes.

Identification of moulds

The filamentous moulds isolated from semen were grown in Sabouraud's Dextrose Agar (Appendix B-7) by point inoculation. The plates were incubated in room temperature and observed daily for rate of growth, general topography, texture, surface pigmentation and 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 then transferred to a microscope slide on which a drop of lactophenol cotton blue was placed. The microscopic

characters of the filamentous fungi was examined first under low power and then under high power magnification.

Antibiotic susceptibility test

Disc diffusion method described by Baur *et al.* (1966) was used for the study. All the isolates were subjected to the antibiotic sensitivity study.

Mueller-Hinton Agar (Himedia laboratories private, limited, Bombay) was prepared. Five colonies from each pure culture were picked up with sterile platimum loop and incubated in five millilitre of nutrient broth at 37°C for 5 hours. The bacterial suspension thus obtained was adjusted with sterile broth, so as to obtain a turbidity comparable to half the density of a McFarland no I standard. This was adsorbed into a sterile cotton swab and uniformly streaked over the entire surface of the agar plate. These plates were allowed to dry for 5 minutes. The different antibiotic discs were then placed on the medium suitably spaced with the help of a flamed and cooled forceps These plates after 18-24 h of incubation at 37°C were examined for the sensitivity pattern.

The diameter of the inhibition zones were measured and recorded for interpretation according to the guidelines of Baur *et al.*, 1966.

Commercial antibiotic discs from Himedia laboratories Private Limited, Bombay were used for this study.

They included ampicillin (10 mcg), amoxycillin (10 mcg), cephaloridine (30 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), cloxacillin (1 mcg), cotrimazine (25 mcg), doxycycline (30 mcg), erythromycin (15 mcg), gentamicin (10 mcg), nitrofurantoin (300 mcg), norfloxacin (10 mcg), oxytetracycline (30 mcg), penicillin (10 units), polymixin-B (300 units), streptomycin (10 mcg), triple sulphas (300 mcg), pefloxacin (5 mcg), furazolidone (50 mcg) and cephalexin (30 mcg).

Antifungal susceptibility test

The study was conducted in the same method as followed for the antibiotic susceptibility test. Sabourauds dextrose Agar was used for smearing the culture The antifungal agents used for the study were nystatin (100 units/disc), amphotericin-B (100 units/disc) and clotrimazole (10 mcg/disc). Discs were applied over the smeared culture plates and incubated for 24 hours. The diameter of the inhibition zone were measured and the susceptibility of the agents to the isolates recorded.

Statistical analysis

The data obtained from the survey of both bacterial and fungal load in frozen semen in three different centres were subjected to statistical analysis as for Snedecor and Cochran (1968). The bulls from which the frozen semen were obtained were grouped into 3 based on their age as follows. (1) The bulls below the age of four years (2) above four years and below six years (3) above six years of age. The mean values of bacterial and fungal load for these age groups were subjected to statistical analysis using student's T test.

The bulls were again divided into three according to their breed - (1) Crossbred Jersey (2) Crossbred Brown-Swiss (3) Crossbred Holstein Friesian. The mean values of bacterial and fungal load in the frozen semen of these bulls were analysed statistically employing student's T test.

Results

RESULTS

The results of the present study to assess the bacterial and fungal load of frozen bull semen and the antibiogram of the isolates are presented in three parts. The first part includes the survey of the microbial flora of frozen semen samples procured from three different centres in Kerala. The second part consist of the results of the characterisation study of the various isolates obtained from frozen semen. The antibiogram of these isolates against different antibiotics which can be added as semen additives, is presented in the final part.

The data obtained from the first part of the study was subjected to statistical analysis as per Snedecor and Cochran (1968).

Survey of the total and specific bacterial load in frozen semen sample procured from different centres

The microbial load per dose of frozen semen collected from the three bull stations in nine different media are presented in Tables 1, 2 and 3.

The mean values of total bacterial load recorded per dose of frozen semen for centres A, B and C were 2260 $\pm$ 880.615,

825.91 ± 186.06 and 80.33 ± 18.47 respectively (Table 4). Statistical analysis using student's 't' test showed that these values were significantly ($P<0.05$) different between the three centres (Table 5a, b). The mean bacterial load per dose of semen produced in Centre A was significantly greater than that produced in Centre B and C. The mean value in Centre B was also significantly greater than that of Centre C (Table 6a, 6b).

The mean values of total fungal load recorded per dose of semen for the Centres A, B and C were 337.5 ± 88.447, 316.36 ± 41.055 and 15.0 ± 3.62 respectively (Table 4). There was no significant difference between the fungal load per dose of semen between Centre A and B. The mean fungal load per dose of semen in Centre A and B was significantly greater than that produced in Centre C ($P<0.05$) (Table 7a, 7b).

Coliforms were not detected in the semen samples of Centre A. The mean coliform counts recorded per straw of frozen semen for Centres B and C were 7.273 ± 3.188 and 8.667 ± 4.43 (Table 4).

The pseudomonads count was detected in two different media - 1. Media for pyocyanin pigment and 2. Media for flourescin pigment. The mean pseudomonads count recorded in the Centres A, B and C in the first media were 527 ± 96.38, 651.82 ± 95.85 and 54.3 ± 19.04 respectively. The corresponding

values in the second media were 725.00 ± 294.52, 596.80 ± 95.056 and 23.67 ± 9.07 respectively (Table 4).

The mean non-lactose fermenters counts recorded in the three centres were 5 ± 5, 5.545 ± 2.280 and 6.00 ± 3.094 respectively. The corresponding values for staphylococci and micrococci were 10.00 ± 5.77, 9.091 ± 4.463 and 2.33 ± 1.609 respectively (Table 4).

All the sample from Centre A were negative for Mycoplasma. While four of the samples from Centre B and two from Centre C were positive for Mycoplasma sp. (Table 1, 2 and 3).

There was not even a single sample totally free of bacteria from Centre A and B, 3 (20%) of samples from Centre C were totally free of any contaminating bacteria.

The minimum and maximum values obtained for total and specific bacterial loads per insemination dose from Centre A were; total count 510 to 3860, Coliforms nil, Pseudomonads 320 to 1600, Staphylococci and Micrococci zero to 20, Non-lactose fermenters zero to 20, and fungi 90 to 510.

The figures for Centre B were total count 160 to 2400, coliforms zero to 35, Pseudomonads 140 to 1020, non-lactose fermenters zero to 25, Staphylococci and micrococci zero to 50 and fungi 125 to 640.

The corresponding values for Centre C were total count 0 to 200, coliforms 0 to 65, pseudomonads 0 to 245, non-lactose fermenters 0 to 40, Staphylococci and micrococci 0 to 20 and fungi 0 to 40 per sample.

The total bacterial count exceeded 500 organisms per insemination dose in all the samples from Centre A, and 72.7 per cent of samples from Centre B. None of the samples from Centre C was found to exceed 500 organisms per straw.

The mean bacterial and fungal count obtained from frozen semen samples of bulls in three different age groups were analysed statistically using students 't' test. The bulls were divided into those aged upto four years, above four years upto six years and above six years. The mean values for these age groups were 595.00 $\pm$ 173.706, 116.667 $\pm$ 33.644 and 1421.429 $\pm$ 625.283 respectively. The mean value for the first age group was significantly higher than the mean values for the second age group. The mean values of the first age group and third age group and also second and third age group did not differ significantly (Table 7a).

The mean fungal load in the semen of the bulls in these age groups were 184.643 $\pm$ 53.281, 83.33 $\pm$ 40.026, 245.00 $\pm$ 76.959 respectively. Statistical analysis revealed that there was no significant difference between the mean fungal load in the semen of bulls included in these age groups (Table 7b).

Similarly, the mean bacterial count per dose of semen from three different crossbred animals were analysed statistically using students t test. The mean values of bacterial count per dose of semen from crossbred Jerseys, crossbred Brown Swiss and crossbred Holstein Friesian bulls were 378.889 ± 145.319, 473.75 ± 152.348 and 1326.875 ± 549.499 respectively. There was no difference between the mean values of semen from crossbred Jerseys and crossbred Brown-Swiss or between crossbred Holstein Friesians and Crossbred Brown Swiss. The mean value in bacterial load of semen of crossbred Holstein Friesians was significantly higher than that of crossbred Jerseys (Table 8a).

The mean fungal load in the semen of these crossbred animals were 98.611 ± 34.556, 307.50 ± 130.982 and 256.25 ± 58.575 respectively. The mean values of crossbred Jerseys and crossbred Brown Swiss animals did not differ significantly and there was no significant difference between the mean values of crossbred Brown Swiss and crossbred Holstein Friesians. As in the case of the mean bacterial load, the mean fungal load of crossbred Holstein Friesians was significantly greater than the mean values of crossbred Jersey bulls ($P<0.05$) (Table 8b).

Typing of bacterial isolates

A total of 59 bacterial isolates obtained from frozen semen were typed upto a genes or species level (Table 9a, 9b and 9c). Of the 59 genes isolates typed, 26 were Gram positives and 33 were Gram negatives. Nine were confirmed as belonging to genus Bacillus, four to genus Corynebacterium, eight to genus Staphylococcus, one to genus Micrococcus, one to genus Aerococcus and three to genus Kurthia. Of the 33 gram negative bacteria seven isolates belonged to genus Alcaligenes, six to genus Pseudomonas, three to genus Flavobacterium, four to genus Escherichia, two to genus Entorobacter, six to genus Citrobacter, two to genus Edwardsiella, one to genus Proteus and two to genus Levinea.

The bacterial isolates from frozen semen samples from Centre A were *Bacillus licheniformis*, *B. cereus*, *Corynebacterium bovis*, *Staphylococcus aureus*, *Aerococcus viridans*, *Flavobacterium*, *Enterobacter aerogenes* and *Escherichia coli* (Table 9a). The bacterial isolates from Centre B were *Bacillus alvei*, *B. brevis*, *B. cereus*, *Corynebacterium pseudodephtheriticum*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Alcaligenes faecalis*, *Flavobacterium* sp., *Pseudomonas alkaligenes*, *Pseudomonas mendocina*, *Edwardsiella tarda*, *Citrobacter koseri* and *Escherichia coli* (Table 9b). The isolates from Centre C

belonged to *Corynebacterium hofmanii*, *Staphyloccous epidermidis*, *Staphylococcus aureus*, *Micrococcus varians*, *Kurthia* sp., *Alcaligenes* sp., *Pseudomonas alkaligenes*, *Levinea* sp., *Enterobacter liquifascians*, *Edwardsiella tarda*, *Citrobacter koseri*, *Citrobacter intermedius*, *Citrobacter freundii*, *Providencia rettgeri* and *Escherichia coli* (Table 9c).

Typing of yeasts and moulds in frozen semen sample

A total of 12 yeasts and eight filamentous moulds were studied. The lone yeast isolate from Centre A was *Candida albicans*. Isolates from Centre B were typed and identified to be belonging to the species, *C. tropicalis* and *C. krusei*. *C. pseudotropicalis* and *C. tropicalis* were isolated from semen samples of Centre C (Table 10).

Of the eight isolates of filamentous mould, *Aspergillus niger*, was obtained from Centre A, *A. fumigatus*, *Microsporum gypseum* and *Penicillium* sp. from Centre B and *A. fumigatus* from Centre C (Table 10).

Antibiotic sensitivity of bacterial isolates

A total of 59 bacterial isolates obtained from frozen semen were subjected to antibiotic sensitivity study against 20 different antibiotics and antibiogram of each isolate was recorded (Tables 11a, b).

Pefloxacin followed by doxycycline, chloramphenicol, ciprofloxacin and norfloxacin were found to be most potent against majority of the isolates. While 94.91 per cent of isolates were susceptible to pefloxacin, 93.23, 93.23, 88.14 and 86.44 per cent of the isolates were susceptible to doxycycline, chloramphenicol, ceprofloxacin and norfloxacin respectively. Only 27.1 per cent and 20.34 per cent of the isolates respectively were sensitive to penicillin and streptomycin, the conventionally used antibiotics in semen extenders (Table 12 and Fig.1).

The other antibiotics, amoxycillin, ampicillin, cotrimazine, cephalexin, cephaloridine, cloxacillin, erythromycin, furozolidone, gentamicin, nitrofurantoin, oxytetracycline, polymixin B and triple sulpha were sensitive to 15.25, 10.17, 64.41, 57.63, 13.56, 22.03, 23.73, 22.03, 61.02, 32.20, 5.08, 72.88 and 74.58 per cent respectively of the isolates tested (Table 12).

Doxycycline and pefloxacin were sensitive to 100 per cent of the gram negative isolates whereas none of the antibiotics were 100 per cent sensitive to the gram positive isolates (Fig.I).

The antibiotics which are sensitive to atleast 50 per cent of the isolates are depicted in the graph.

Antifungal sensitivity of the isolates

Twelve yeasts obtained from frozen semen were subjected to antifungal sensitivity study. Three antifungal agents viz., Nystatin, Amphotericin B and Clotimazole were tested for sensitivity.

None of the antifungal agents tested were 100 per cent sensitive to the yeasts isolated. Nystatin was sensitive to 90.9 per cent, clotrimazole to 81.80 per cent and Amphotericin B to 36.3 per cent of the isolates (Fig.2).

Table 1. Microbial count per dose of frozen semen collected from bull station, Mannuthy, in different media

Bull No.	Breed	Date of birth	Blood agar	EMB	McC	MHA	MSA	PsPy	PsFl	PDA 37°C	PDA 22°C	PPLO
204 (a)*	CBHF	16.9.87	510	-	10 LF	500	20	380	320	300	80	-
204 (b)*	CBHF	16.9.87	3860	-	40 LF	940	-	480	470	90	-	-
215 (c)*	CBHF	16.6.87	980	-	-	900	-	810	510	340	30	-
215 (b)*	CBHF	16.6.87	3690	-	20 LF 20 NF	420	20	440	1600	470	40	-

(a) and (b) are semen collections on different days

LF - lactose fermenters
NF - Non-lactose fermenters
EMB - Eosin Methylene Blue Agar
McC - McConkey's Agar
MHA - Mueller hinton Agar
MSA - Mannitol Salt Agar
PsPy - Pseudomonas Agar for Pyocyanin
PsFl - Pseudomonas Agar for Flourescin
PDA - Potato Dextrose agar
PPLO - Pleuro Pneumonia like organism agar (Mycoplasma agar)

Table 2. Microbial count per dose of frozen semen collected from bull station - Dhoni, in different media

Bull No.	Breed	Date of birth	Blood agar	EMB	McC	MHA	MSA	PsPy	PsFl	PDA		PPLO
										37°C	22°C	
KA 4354	CBJ	2.10.92	2400	5	-	800	10	645	710	175	15	-
KA 3827	CBJ	28.5.91	995	-	85 LF 5 NF	565	15	1010	900	270	20	-
KA 2088	CBJ	28.4.87	910	5	150 LF	165	15	635	430	320	25	50
KA 4087	CBJ	9.9.91	1000	10	5 LF 10 NF	170	-	760	1040	460	-	-
KA 4305	CBJ	5.4.92	580	35	10 LF 5 NF	515	-	1020	900	290	-	5
KA 4219	CBBS	3.10.92	775	15	85 LF 25 NF	400	50	540	530	640	-	-
KA 4360	CBBS	26.8.92	675	-	30 LF	225	5	285	220	290	20	-
KA 2848	CBBS	24.1.90	325	-	15 LF	180	5	235	245	265	15	30
KA 3884	CBHF	1.6.91	1085	-	50 LF	680	-	855	550	230	30	-
KA 3472	CBHF	6.6.90	160	10	15 LF 5 NF	515	-	1020	900	290	-	5
KA 3758	CBHF	23.1.91	180	-	30 LF 1 NF	125	-	165	140	120	5	-

Table 3. Microbial load per dose of frozen bull semen collected from bull station - Mattupetti in different media

Bull No.	Breed	Date of birth	Blood agar	EMB	McC	MHA	MSA	PsPy	PsFl	PDA		PPLO
										37°C	22°C	
KA 4080(a)	CBJ	10.5.89	95	5	20 LF 40 NF	70	-	100	70	5	5	30
KA 4080(b)	CBJ	10.5.89	35	10	-	-	-	5	-	-	10	-
KA 3987(a)	CBJ	20.7.91	200	-	10 NF	35	20	90	35	35	-	-
KA 3987(b)	CBJ	20.7.91	155	-	-	10	-	25	-	35	5	-
KA 3987(c)	CBJ	20.7.91	25	20	-	-	-	-	-	-	-	-
KA 4009(a)	CBJ	22.8.91	200	-	-	35	-	245	35	35	-	15
KA 4009(b)	CBJ	22.8.91	90	65	10 LF 25 NF	70	15	180	30	10	-	-
KA 3638	CBJ	18.10.90	85	-	20 LF 15 NF	-	-	-	-	10	-	-
KA 3718	CBJ	25.12.90	20	10	5 NF	-	-	10	-	5	-	-
KA 3356	CBJ	25.4.89	-	-	-	-	-	-	-	-	20	-
KA 4301	CBJ	27.3.92	30	20	-	-	-	55	-	-	25	-
KA 3987	CBJ	27.6.88	-	-	-	-	-	-	-	-	-	30
KA 3864	CBJ	29.7.90	-	-	-	-	-	-	-	-	-	-
KA 4410	CBBS	18.1.93	120	-	-	20	-	70	105	-	-	-
KA 3169	CBHF	24.5.89	150	-	-	105	-	35	80	25	-	-

(a), (b) and (c) are semen collections on different days

Table 4. Mean of total and specific bacterial load per straw from different centres of frozen semen

Centre	Blood agar	EMB	Non lactose fermenters	MHA	MSA	PsPy	PsFl	PDA
A	2260 ± 80.615	0	5 ± 5	690 ± 134.04	10 ± 5.774	527.5± 96.382	725 ± 294.52	337.5 ± 88.447
B	825.91 ± 186.066	7.23 ± 3.188	4.545 ± 2.282	391.82 ± 69.949	9.091 ± 4.463	651.82 ± 95.846	596.818 ± 95.056	316.364± 41.055
C	80.33 ± 18.468	8.667 ± 4.431	6.0 ± 3.094	23 ± 8.685	2.33 ± 1.609	54.33 ± 19.042	23.667 ± 9.069	15.0 ± 3.619

Table 5a. Student's 't' test on the mean total bacterial count in different stations

Centre	Mean total bacterial count	t value
Centre A	2260.00 ± 880.615	2.2197
Centre B	825.91 ± 186.066	

The means differ significantly P<0.05

Centre	Mean total bacterial count	t value
Centre A	2260.00 ± 880.615	3.5918
Centre C	80.33 ± 18.468	

The means differ significantly P<0.05

Centre	Mean total bacterial count	t value
Centre B	825.91 ± 186.066	5.8108
Centre C	80.33 ± 18.468	

The means differ significantly P<0.05

Table 5b. Student's t test on the mean bacterial load in semen from different stations

Station	Mean bacterial load per dose of semen
Centre A	2260.00 ± 880.615[a] (4)
Centre B	825.91 ± 186.066[a] (11)
Centre C	80.33 ± 18.468[a] (15)

Means with one superscript in common differ significantly ($P<0.05$)

Figures in parenthesis indicate number of observations

Table 6a. Student's 't' test on the mean fungal load in in different stations

Centre	Mean fungal load per dose of semen	t value
Centre A	337.500 ± 88.447	0.1056
Centre B	316.364 ± 41.055	

The means does not differ significantly P<0.05

Centre	Mean fungal load per dose of semen	t value
Centre A	337.50 ± 88.447	5.0058
Centre C	15.00 ± 3.619	

The means differ significantly P<0.05

Centre	Mean fungal load per dose of semen	t value
Centre B	316.364 ± 41.055	12.1864
Centre C	15.000 ± 3.619	

The means differ significantly P<0.05

Table 6b. Student's t test on the mean fungal load in semen from different stations

Station	Mean fungal load per dose of semen
Centre A	337.500 ± 88.447[a] (4)
Centre B	316.364 ± 41.055[b] (11)
Centre C	15.000 ± 3.619[ab] (15)

Means with one superscript in common differ significantly ($P<0.05$)

Figures in parenthesis indicate number of observations

Table 7a. Student's t test on the mean bacterial load in semen of bulls in different age group

Age group of bulls	Mean bacterial load per dose of semen
Upto 4 years	595.000 ± 173.706[a] (14)
4.1 year to 6 years	116.667 ± 33.644[ab] (9)
Above 6.1 years	1421.429 ± 625.283[c] (7)

Means with one superscript in common differ significantly (P<0.05)

Table 7b. Student's t test on the mean fungal load in semen of bulls in different age group

Age group of bulls	Mean fungal load per dose of semen
Upto 4 years	184.643 ± 53.281[a] (14
4.1 year to 6 years	83.338 ± 40.026[b] (9)
Above 6.1 years	245.000 ± 76.959[c] (7)

Means with one superscript in common differ significantly (P<0.05)

Table 8a. Student's t test on the mean bacterial load in semen of bulls in different breeds

Breed	Mean bacterial load per dose of semen
CBJ	378.889 ± 145.319[a] (14)
CBBS	473.750 ± 152.348[b] (4)
CBHF	1326.875 ± 549.499[ac] (8)

Means with one superscript in common differ significantly ($P<0.05$)

Table 8b. Student's t test on the mean fungal load in semen of bulls in different breeds

Breed	Mean fungal load per dose of semen
CBJ	98.611 ± 34.556[a] (14)
CBBS	307.500 ± 130.982[b] (4)
CBHF	256.250 ± 58.575[ac] (8)

Means with one superscript in common differ significantly ($P<0.05$)

Table 9a. Bacterial organisms isolated from semen samples of Mannuthy bull station

Isolate	Species
M 3	*Bacillus licheniformis*
M 4	*Escherichia coli*
M 10	*Bacillus cereus*
M 22	*Enterobacter aerogenes*
M 23	*Bacillus cereus*
M 24	*Bacillus cereus*
M 25	*Corynebacterium bovis*
M 26	*Flavobacterium* sp.
M 27	*Bacillus cereus*
M 29	*Aerococcus viridans*
M 30	*Staphylococcus aureus*

Table 9b. Bacterial organisms isolated from semen samples of Dhoni bull station

Isolate	Species
D 2	*Staphylococcus epidermidis*
D 5	*Alcaligenes faecalis*
D 6	*Alcaligenes faecalis*
D 7	*Edwardsiella tarda*
D 8	*Pseudomonas aloalegenes*
D 10	*Staphylococcus epidermidis*
D 11	*Bacillus alvei*
D 13	*Bacillus alvei*
D 15	*Alkaligenes* sp.
D 18	*Ps. alcaligenes*
D 21	*Staph epidermidis*
D 22	*Alcaligenes* sp.
D 23	*Staphylococcus aureus*
D 24	*Corynebacterium pseudodiphtheriticum*
D 25	*Alcaligenes faecalis*
D 27	*Citrobacter koseri*
D 30	*Pseudomonas alkaligenes*
D 34	*Bacillus brevis*
D 35	*Bacillus cereus*
D 39	*Flavobacterium* sp.
D 41	*Pseudomonas mendocina*
D 42	*Flavobacterium* sp.
D 44	*Escherichia coli*
D 47	*Pseudomonas mendocina*

Table 9c. Bacterial organisms isolated from semen samples of Mattupetty bull station

Isolate	Species
K 2	*Corynebacterium hofmanii*
K 4	*Kurthia* sp.
K 5	*Kurthia* sp.
K 11	*Levinea* sp
K 12	*Enterobacter liquifascians*
K 13	*Micrococcus varians*
K 14	*Citrobacter koseri*
K 15	*Alcaligenes* sp.
K 17	*Citrobacter intermedius*
K 19	*Edwardsiella tarda*
K 20	*Kurthia* sp.
K 22	*Citrobacter intermedius*
K 23	*Citrobacter freundii*
K 24	*Corynebacterium hofmanii*
K 25	*Citrobacter freundii*
K 26	*Levinea* sp.
K 27	*Staphylococcus epidermidis*
K 28	*Providencia rettgeri*
K 29	*Escherichia coli*
K 30	*Staphyloccus aureus*
K 31	*Pseudomonas alkaligenes*
K 32	*Escherichia coli*
K 33	*Staph. epidermidis*
K 36	*Alcaligenes faecalis*

Table 10. Fungal organisms isolated from semen samples of different bull stations

Station	Isolate no.	Species
Mannuthy	M 31	*Candida albicans*
	M 28	*Aspergillus niger*
Dhoni	D 31	*Candida tropicalis*
	D 38	*Candida tropicalis*
	D 40	*Candida tropicalis*
	D 45	*Candida krusei*
	D 4	*Aspergillus fumigatus*
	D 36	*Penicillium* sp.
	F 1	*Aspergillus fumigatus*
	F 3	*Penicillium* sp.
	F 5	*Microsporum gypseum*
Mattupetti	K 1	*Candida pseudotropicalis*
	K 3	*Candida tropicalis*
	K 7	*Candida pseudotropicalis*
	K 8	*Candida pseudotropicalis*
	K 9	*Candida pseudotropicalis*
	K 10	*Candida pseudotropicalis*
	K 6	*Aspergillus fumigatus*
	K 21	*Aspergillus fumigatus*

Table 11a. Antibiotic sensitivity pattern of the Gram Negative Isolates

Isolates	Antibiotics																			
	Am	A	C	Cf	Cm	Cp	Cr	Cx	Do	E	Fr	G	Nf	Nx	O	Pf	P	Pb	S	Sz
M 4	R	S	S	S	S	R	R	R	S	S	R	S	S	R	R	S	S	S	R	S
M 22	R	R	S	R	R	S	R	R	S	R	R	R	S	S	R	S	R	R	S	S
M 26	S	R	S	S	S	R	R	S	S	S	R	S	S	R	R	S	S	S	S	S
D 5	R	R	R	S	R	R	R	R	S	R	R	R	R	S	R	S	R	S	R	R
D 6	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	S	R	R	S
D 7	R	R	S	S	R	R	R	R	S	R	R	R	R	S	R	S	R	S	R	R
D 8	R	R	S	S	S	R	R	R	S	R	R	R	R	S	R	S	R	R	R	R
D 15	R	R	S	S	S	R	R	R	S	R	R	R	R	S	R	S	R	S	R	R
D 18	R	R	S	S	S	S	R	R	S	S	R	S	R	S	R	S	S	R	R	S
D 22	R	R	S	S	R	R	R	R	S	R	R	S	R	S	R	S	R	S	R	R
D 25	R	R	S	S	S	S	R	R	S	S	R	S	R	S	R	S	R	S	R	S
D 27	R	R	S	S	S	R	R	R	S	R	R	R	S	S	R	S	R	S	R	S
D 30	R	R	S	R	R	S	R	R	S	R	R	S	R	S	R	S	R	S	R	S
D 39	R	R	S	S	S	S	S	R	S	R	R	S	R	S	R	S	S	S	R	S
D 41	R	R	S	S	S	R	R	R	S	R	R	R	R	R	R	S	R	S	R	S
D 42	S	R	S	R	R	S	S	R	S	R	R	S	R	S	R	S	S	S	R	S

Contd.

Table 11a. (Contd.)

Isolates	Antibiotics																			
	Am	A	C	Cf	Cm	Cp	Cr	Cx	Do	E	Fr	G	Nf	Nx	O	Pf	P	Pb	S	Sz
D 43	R	R	S	S	S	R	R	R	S	R	R	S	R	S	R	S	R	R	R	S
D 47	R	R	S	S	S	R	R	R	S	R	R	R	R	R	R	S	R	S	R	S
K 11	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 12	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 14	R	R	S	S	R	S	R	R	S	R	R	S	R	S	R	S	S	R	R	S
K 15	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 17	R	R	S	S	S	S	R	R	S	S	R	S	R	S	R	S	R	S	R	S
K 19	R	R	S	S	S	R	R	R	S	S	R	S	S	S	R	S	R	R	R	R
K 22	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 23	R	R	S	S	S	R	R	R	S	R	S	S	R	S	R	S	R	S	R	S
K 25	R	R	S	S	R	R	R	R	S	R	R	S	R	S	R	S	R	S	R	S
K 26	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 28	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 29	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	R	R	S
K 31	R	R	S	S	R	S	R	R	S	R	R	S	S	S	R	S	R	R	R	R
K 32	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 36	S	R	S	S	S	S	R	S	S	S	S	S	R	S	R	S	S	S	R	S

S - Sensitive R - Resistant

Table 11b. Antibiotic sensitivity pattern of the Gram Positive Isolates

Isolates	Antibiotics																			
	Am	A	C	Cf	Cm	Cp	Cr	Cx	Do	E	Fr	G	Nf	Nx	O	Pf	P	Pb	S	Sz
M 3	R	R	S	S	R	R	R	R	S	R	R	S	S	S	R	S	R	R	S	S
M 10	R	S	S	S	R	S	R	R	S	R	S	S	S	S	R	S	R	R	R	R
M 23	S	S	R	R	R	R	R	R	S	R	S	S	R	S	R	S	S	S	R	R
M 24	R	R	S	S	R	R	R	R	S	R	S	S	S	S	R	S	R	R	R	S
M 25	S	R	S	S	R	R	S	R	S	S	R	S	R	S	R	S	R	R	R	S
M 27	R	R	S	S	R	R	R	R	R	R	S	S	S	S	R	S	R	R	R	S
M 29	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	S	R	R
M 30	R	R	S	R	S	R	R	R	S	R	R	S	S	R	R	R	R	R	R	R
D 2	R	S	S	S	S	S	S	S	S	S	R	S	R	R	S	R	S	S	S	S
D 10	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
D 11	S	S	S	S	S	S	R	S	S	S	S	S	R	S	R	S	S	S	R	S
D 13	R	R	S	S	R	R	R	R	S	S	R	S	S	R	R	S	S	S	R	S
D 21	R	R	S	S	S	S	S	S	S	R	R	S	R	S	S	S	R	S	S	S
D 23	R	R	S	S	S	S	R	S	S	R	S	S	S	S	R	S	R	S	S	S
D 24	R	R	S	S	R	R	R	S	S	R	S	R	R	S	R	S	S	S	S	R

Contd.

Table 11b. (Contd.)

Isolates	Antibiotics																			
	Am	A	C	Cf	Cm	Cp	Cr	Cx	Do	E	Fr	G	Nf	Nx	O	Pf	P	Pb	S	Sz
D 34	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S
D 35	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	S	R	S
K 2	R	R	S	S	S	S	R	R	S	R	R	S	R	S	S	S	R	S	R	S
K 4	R	R	S	S	S	S	R	R	S	S	R	S	R	S	R	S	R	R	S	S
K 5	R	R	S	S	S	S	R	R	S	R	R	S	R	S	R	S	R	S	R	S
K 13	R	R	S	S	R	S	S	S	S	R	R	S	S	S	R	S	S	S	S	S
K 20	R	R	S	S	S	S	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 24	R	R	S	S	S	R	R	R	S	R	R	R	R	S	R	S	R	S	R	S
K 27	R	R	S	S	R	R	R	S	S	R	S	S	S	S	R	S	R	S	R	R
K 30	S	R	S	S	S	S	R	S	S	R	S	R	S	S	R	S	R	S	R	R
K 33	R	R	S	S	S	S	R	S	R	R	R	S	S	S	R	S	R	S	S	R

S - Sensitive R - Resistant

Table 12. Sensitivity percentage of different antibiotics

Antibiotics	Percentage of isolate sensitive		Percentage of total isolates sensitive
	Gram negative	Gram positive	
Amoxycillin	9.09	23.08	15.25
Ampicillin	3.03	19.23	10.17
Chloramphenicol	96.97	88.46	93.23
Ciprofloxacin	90.91	84.62	88.14
Cotrimazine	72.73	53.84	64.41
Cephalexin	57.58	57.69	57.63
Cephaloridine	6.06	23.08	13.56
Cloxacillin	6.06	42.31	22.03
Doxycycline	100.00	84.62	93.23
Erythromycin	21.21	26.92	23.73
Furazolidone	6.06	42.31	22.03
Gentamicin	48.48	76.92	61.02
Nitrofurantoin	18.18	50.00	32.20
Norfloxacin	87.88	84.62	86.44
Oxytetracycline	0.00	11.54	5.08
Pefloxacin	100.00	88.46	94.91
Penicillin-G	24.24	30.77	27.10
Polymixin-B	72.73	73.08	72.88
Streptomycin	6.06	38.46	20.34
Triple sulphas	78.79	69.23	74.58

Fig.1 ANTIBIOTIC SENSITIVITY PATTERN OF THE ISOLATES

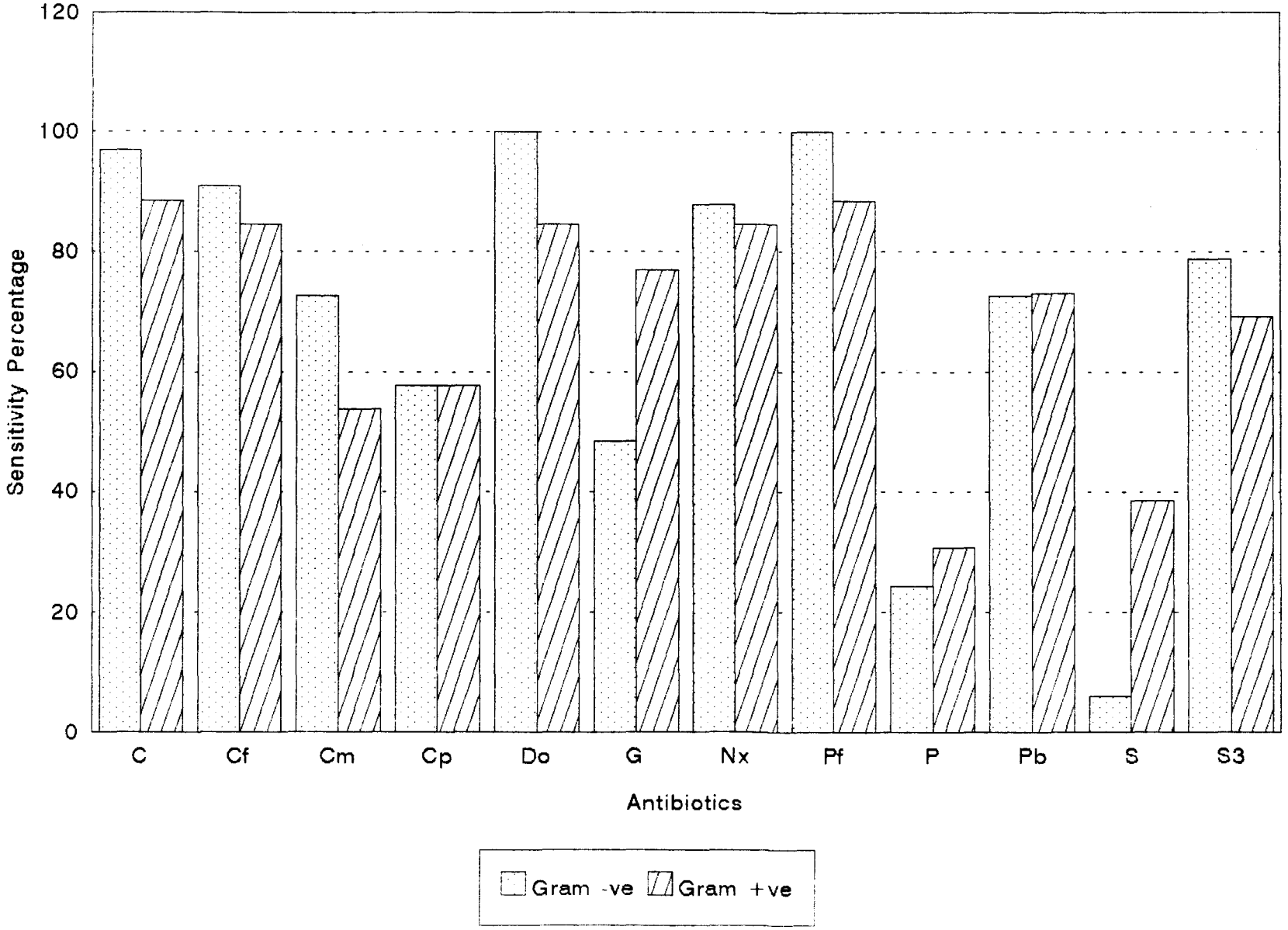
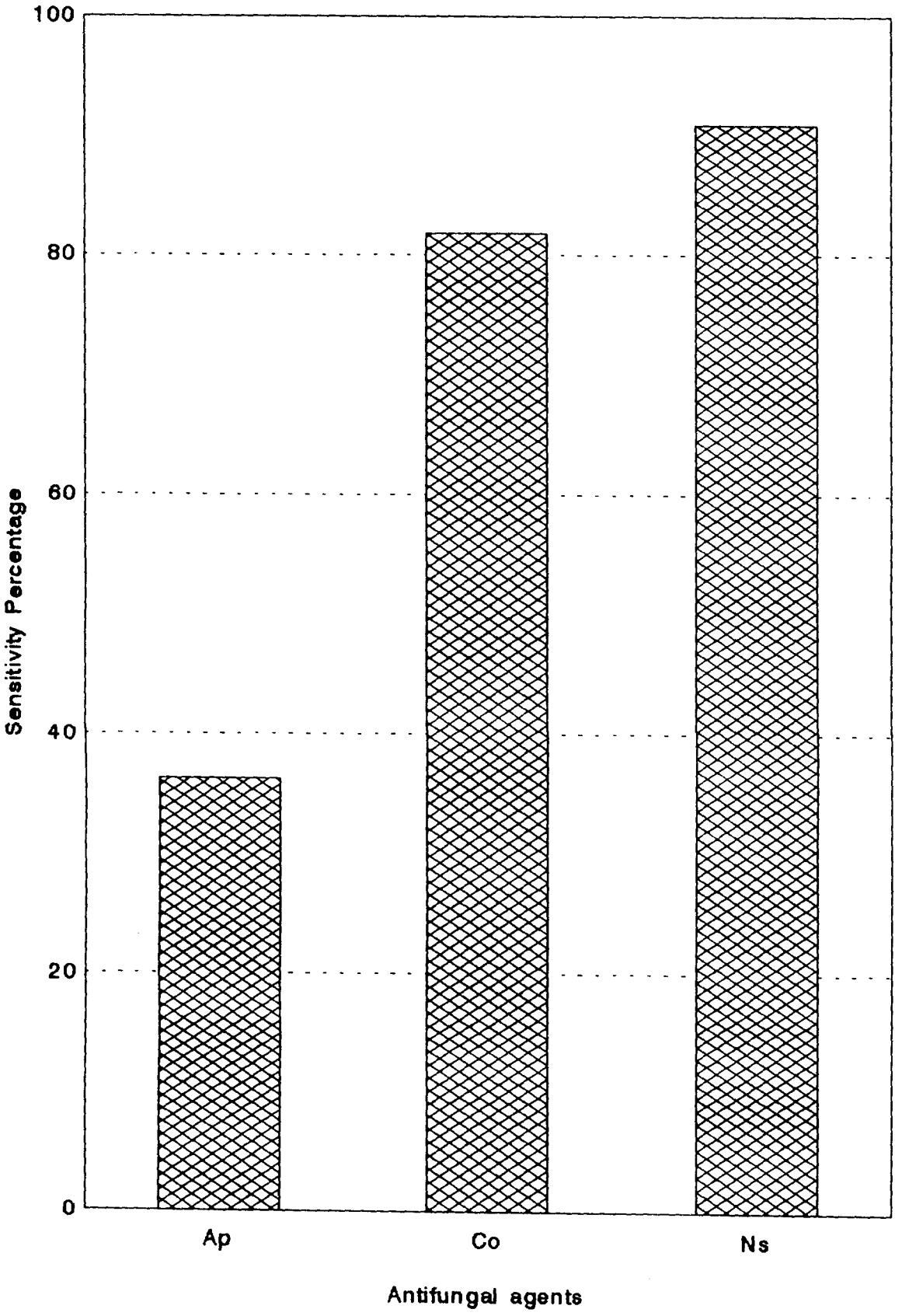


FIG. 2 ANTIFUNGAL SENSITIVITY PATTERN OF THE ISOLATES



Discussion

DISCUSSION

Semen samples procured from three artificial insemination centres in Kerala were examined for the presence of contaminating bacteria and fungi in this study. The mean values of bacterial load in frozen semen from the three centres A, B and C were 2260 ± 880.615, 825.9 ± 186.06 and 80.33 ± 18.47 respectively (Table 1, 2, 3). The values for centre A was significantly higher than the other two centres eventhough the diluent used and the procedures followed for freezing were similar in the three centres. The total bacterial loads were estimated per straw basis irrespective of the volume of the straw. Straws from centre A contained 0.5 millilitre of semen and that from centres B and C contained 0.25 ml semen. The calculation was done on this basis in order to find out the extend to which this centres fulfilled the ISI specifications which directed each frozen semen production centres to limit the number of bacterial organism to 500 per dose (ISI, 1976). From the present study it was shown that only centre C produced semen which confirm to the standards laid by the ISI. At the same time in centre A, 100 per cent and in Centre B, 72.72 per cent of the samples exceeded the ISI specification.

The proposal made by Ostasczko (1976) to the international organisation of biological standards that the

limit of saprophytic organism per millilitre of frozen semen be limited to 500 organisms was not accepted (Wierzbowski, 1981). Eventhough the limit of 500 organisms per millilitre of frozen semen was not accepted, the limits of 500 organism per dose of frozen semen was accepted by the international organisation of biological standards (Raghavan *et al.*, 1982).

Romaniuk (1965) reported that the count of 300,000 or more per ml of semen could adversely affect fertility rate. In the present study, the maximum recorded number of organisms per millilitre of semen was 9600 (2400/0.25 ml insemination dose) in an animal from centre B. Most of the other samples had counts much below this level (Table 1, 2, 3). Nimai Singh *et al.* (1990) found that the mean bacterial load in the semen, 90 days post freezing was $2.5 \pm 0.16 \times 10^3$ per millilitre. Most of the literature concerning the number of bacteria in frozen semen gives the figures in terms of organisms per millilitre. When the mean values obtained per dose of semen were converted into organisms per millilitre the corresponding counts were 4520 ± 1761.23, 3303.64 ± 744.24 and 321.32 ± 73.88 respectively for centres A, B and C The value obtained were almost similar or lower than those obtained by earlier workers (Pospelov *et al.*, 1973), Wierzbowski *et al.*, 1973; Wierzbowski and Szmyd, 1976; Nowakowski and Wierzbowski, 1980).

Kher and Dholakia (1984) reported that the bacterial contamination of semen varied according to the season, the load being maximum during summer and the minimum during winter. In the present study the samples from centre A, B and C were collected and frozen during November to April 1994-95. The maximum and minimum temperatures recorded during this period in Centre A and Centre B were similar being 35°C and 20°C. Centre C is located more than 3000 feet above sea level where the temperature seldom rise above 20°C, during the study period. The significantly lower number of contaminant bacteria in semen from centre C can be attributed to the lower ambient temperature in the region.

The mean bacterial load in Centre A was significantly higher than that from the other two Centres ($P<0.05$) (Table 4a, 5b) and between the three centres the count was significantly different. This difference can be attributed to experimental nature of the freezing carried out in Centre A and the differences in the relative degree of hygienic practices followed and the sanitary environment provided in these centres. Wierzbowski (1981) reported that management practices followed at different centres reflected on the bacterial load in semen produced by those centres. The animals in Centre A were all above six years of age and this may be a contributory factor for the significantly higher mean

value of bacterial load in the frozen semen samples from this centre.

The minimum and maximum bacterial load in frozen semen recorded in Centre A, B and C were 510 to 3860, 160 to 2400 and 0 to 200 respectively. The corresponding values for the fungal load were 90 to 510, 125 to 640 and 0 to 40 respectively per sample.

There is total paucity of literature on the relative load of different types of bacteria present in the frozen semen. In the present study, the mean coliform load, in the three centres were zero, 7.273 ± 3.188 and 8.667 ± 4.43 respectively and the maximum recorded number was only 65 organisms (260 per millilitre) per dose (Centre C) (Table 1, 2, 3, 4). Boryczko (1982) demonstrated that a concentration of 30 million organisms of *E. coli.*, *Pseudomonas aeroginosa* and *Staphylococcus aureus* per millilitre causes reduction in the percentage of live spermatozoa. The mean pseudomonads count obtained in the media for pyocyanin in the three centres were 527 ± 96.38, 651.82 ± 95.85 and 54.3 ± 19.04 per dose respectively and that in the media for flouresin were 725 ± 294.52, 596.8 ± 95.056 and 23.67 ± 9.07 respectively (Table 4). The maximum number of pseudomonads detected was 1600 per dose of semen (3200 per millilitre) from Centre A. The mean values of staphylococcus and micrococcus in the semen

from Centre A, B and C were 10.0 ± 5.774, 9.091 ± 4.463 and 2.33 ± 1.609 respectively and the maximum recorded number was 50 per insemination dose (200/millilitre) from Centre B (Table 2). These values are also below the concentration suggested by Boryczko (1982) as detrimental to spermatozoa.

All the samples from Centre A were free of mycoplasma contamination while four of the sample (36.36%) from Centre B and two (13.33) from Centre C contained mycoplasma Mycoplasma species were isolated from the semen of bulls by several workers Lein and Nielsen, 1975; Juramanova *et al.*, 1977; Ungureanu *et al.*, 1984; Fejes, 1986; Machado and Atalia, 1987; Marciczewska, 1987; Rosario-gonsalves, 1990). Erno (1975) reported that 7.8 per cent of the semen samples from Danish Artificial Insemination bulls contained mycoplasmas. Juramanova and Sterbanova (1977) observed a relationship between inferior quality of the ejaculates and the concentration of mycoplasma. Mycoplasma organism was present in nine samples of frozen semen from 45 bulls (Fish *et al.*, 1985). Fejes (1986) points out that the semen quality of the mycoplasma carriers was usually inferior.

The mean bacterial count of semen from bulls belonging to three age groups were 595.00 ± 173.706, 116.667 ± 33.644 and 1421.429 ± 625.283 respectively (Table 7a). The bacterial count per dose of semen obtained for the first age group

(bulls below four years of age) was significantly higher than the second age group (between four and six years of age) ($P<0.05$). The majority of the bulls below four years of age will be in the training period of semen collection. During this period the penile shaft is inserted into the artificial vagina more than once before ejaculation. If in any case, the same artificial vagina is given a second time, it can lead to contamination. In older bulls also the ejaculation will not always take place in the first instance. Here also a repeatedly used artificial vagina can lead to contamination of the semen. Romaniuk *et al.* (1971) observed that as the age of bulls increases, the bacterial count also increases. In younger bulls below the age of four years the count was low. In the present study the value in older bulls were high. In the younger bulls also it was significantly higher than the bulls in the age group of four to six years. Kher and Dholakia (1984) reported a higher bacterial count in the semen of bulls above six years of age.

The mean bacterial count per dose of semen from crossbred Jerseys, crossbred Brownswiss, and crossbred Holstein Friesians were 378.889 $\pm$ 145.319, 473.75 $\pm$ 152.348 and 1326.875 $\pm$ 549.499 respectively (Table 8a). The corresponding values for the mean fungal load per dose of semen was 98.611 $\pm$ 34.556, 307.5 $\pm$ 130.982 and 256.25 $\pm$ 58.575, respectively (Table 8b). Both the mean bacterial and fungal load per dose

of semen from crossbred Holstein Friesians were greater than that of crossbred Jerseys. Kher and Dholakia (1984) in their studies had reported that during the summer season the bacterial load per millilitre of fresh semen varied in different breeds of cattle. Sixty two per cent crossbred Holstein Friesians in the present study were in the age group of above six years and this can be a reason for increased count in the semen of these animals.

The mean fungal load per dose of semen in the three centres were 337.5 ± 88.447, 316.36 ± 41.055 and 15.0 ± 3.62 respectively (Table 4). The mean fungal load per dose of semen in the Centre A and B was significantly greater than that produced in Centre C ($P<0.05$) (Table 6a). The minimum and maximum load obtained per dose of semen in the three centres were 90 to 510, 125 to 640 and 0 to 40 respectively. All the samples from Centre A and B contained fungal organisms whereas eleven out of 15 (73.33%) samples from Centre C contained these organisms. Horter (1962) examined preputial samples from 293 bulls for fungi of which only 38 (13%) yielded fungi. Thirty six per cent of the cultured preputial washings yielded fungi (Rob and Toman, 1970). They also observed that the centres with the highest contamination had the lowest fertility. In the present study the fungal load in semen from all the centres denote an inferior quality of semen. The cause of such a figure can be attributed to the

lack of hygienic practices followed in these centres. This highlights the need for the addition of an antifungal agent in the semen extender.

Typing of bacterial isolates

The wide spread presence of certain microorganisms in the environment of cattle can pose a potential venereal problem when they contaminate bull semen. Bacterial organisms obtained from frozen semen procured from Centre A belonged to the species *Bacillus cereus, B. licheneformis, Corynebacterium bovis, Staphylococcus aureus, Aerococcus viridans, Flavobacterium* sp., *Enterobacter aerogenes* and *Escherichia coli*. Bacterial speciess isolated from Centre B were *Bacillus alvei, B. brevis, B. cereus, Corynebacterium pseudodephtheriticum, Staphylococcus epidermidis, Staphylococcus aureus, Alcaligenes faecalis, Flavobacterium* sp., *Pseudomonas alkaligenes, Pseudomonas mendocina, Edwardsiella tarda, Citrobacter koseri* and *Escherichia coli*. The isolates from Centre C belonged to *Corynebacterium hofmanii, Staphyloccous epidermidis, Staphylococcus aureus, Micrococcus varians, Kurthia* spp., *Alcaligenes* sp., *Pseudomonas alkaligenes, Levinea* sp., *Enterobacter liquifasclans, Edivardsiella tarda, Citrobacter koseri, C. intermedius, C. freundi, Providencia rettgeri* and *Eschierichia coli* (Tables 9a, 9b, 9c).

The result of the present characterisation study agree with that of several earlier workers (Blom and Dam, 1966; Marinov *et al.*, 1966; Reddy *et al.*, 1971; Pospelov *et al.* 1973; Brown *et al.*, 1979; Kirchhelle, 1975; Meyer *et al.*, 1980; Nowakowski *et al.*, 1980; Saikia *et al.*, 1987; Danowski, 1989; Naidu *et al.*, 1981, Rahman *et al.*, 1983, Gangadhar *et al.*, 1986 and Eaglesome *et al.*, 1992).

Although most of these organisms are non-pathogenic, some are opportunistic pathogens such as *Pseudomonas aeroginosa*, *Corynebaterium* sp., *Staphylococcus* sp., *Streptococcus* sp., *E. coli*, *Proteus* sp. and certain moulds and yeasts (Eaglesome *et al.*, 1992).

Romaniuk (1965) demonstrated that conception rates were adversely affected only when the bacterial count exceeded 3000,000 per millilitre. However other studies have indicated that opportunistic organisms may affect the functional ability of spermatozoa (Meyer *et al.*, 1980; Nowakowski *et al.*, 1980). Meyer *et al.* (1980) demonstrated the damage of spermatozoa by the extracellular proteins of *Staphylococcus aureus*. This organism had been isolated from semen from all the three centres. Pseudomanads were isolated from Centre B and Centre C. *Pseudomonas aeroginosa* as described by Getty and Ellis (1967) can lead to development of metritis, cervicitis and vaginitis in heifers inseminated with semen containing this

organism. Aleem *et al.* (1990) observed that all the strains of *Staphylococcus aureus*, 50 per cent of *E. coli* and all the strains of Pseudomonas were lethal to mice after intraperitoneal injection These three species were isolated from the semen of all the centres. However, Radchankov *et al.* (1990) reported that insemination of the female rabbit with semen artificially contaminated with *Pseudomonas aeroginosa* did not impair embryonic survival. Radchankov *et al.* (1993) also observed that the insemination index did not differ significantly between semen samples positive or negative for *Pseudomonas aeroginosa*.

Of the various species of bacteria isolated (Table 9a, b, c) *B. cereus*, *Corynebacterium bovis*, *Aerococcus viridans*, *Staphylococcus epidermidis*, *Edwardsiella tarda*, *Citrobacter freundii*, and *Providencia rettgeri* are regarded as opportunistic pathogens (Buchanan and Gibbons, 1974).

Typing of fungal isolates

Eleven isolates of yeast were characterised upto the species level. *Candida albicans* was isolated from Centre A. Semen from Centre B contained *Candida tropicalis* and *Candida krusei*. *Candida pseudotropicalis* and *Candida tropicalis* were isolated from semen samples of Centre C (Table 10).

Eight isolates of filamentatous fungi from semen included *Aspergillus niger* from Centre A, *Aspergillus fumigatus*, *Microsporum gypseum* and *Penicillium* sp. from Centre B and *A. fumigatus* from Centre C (Table 10).

Yeasts and moulds belonging to these species were isolated by earlier workers (Horter, 1962; Zvareva and Repco (1968); Richard *et al.*, 1976, Gangadhar *et al.* (1986), Natalia, 1987).

Yeasts are not part of the natural flora of bull semen but enter extended semen during processing where they may multiply unchecked by antibiotics which are routinely included in semen extenders.

C. albicans is a part of the normal flora of the animal body and is an opportunistic fungus that causes candidiasis in compromised animals. All the other species of candida isolated in the present study are also opportunistic pathogens.

Aspergillus niger, *A. fumigatus* and *Penicillium* sp. are also opportunistic pathogens. These organisms were isolated from cases of abortion and repeat breeding in crossbred animals (Misra *et al.*, 1984). They had also isolated *C. albicans* and *C. tropicalis*.

Antibiotic sensitivity of bacterial isolates

From among the twenty antibiotics studied for sensitivity to 59 isolates of bacteria obtained from frozen semen samples, pefloxacin followed by doxycycline chloramphenicol, ciprofloxacin and norfloxacin were the most effective antibiotics against majority of the isolates. In the present study 94.91, 93.23, 93.23, 88.14 and 86.44 per cent of bacteria were sensitive to pefloxacin, doxycycline, chloramphenicol, ciprofloxacin, and norfloxacin respectively (Table 12). Gentamicin was sensitive to only 61.02 per cent of the isolates. However, Kurudshiiski (1980) reported that all the isolates from bull semen were resistant to chloramphenicol but all were sensitive to gentamicin. Gentamicin was also reported to be sensitive to all the isolates by Naidu (1981), although the resistance pattern observed for tetracyclines and ampicillin was similar to the present study.

Chloramphenicol and gentamicin were reported to be the highly sensitive antibiotics against semen contaminants (Saikia *et al.*, 1987, Nimai Singh, 1990). Wayda (1981) suggested a combination of gentamicin and penicillin for the preparation of diluent and cryopreservation.

Seventy five day non return rate was observed to be 72.3 per cent when semen diluted in Tris-egg yolk extender containing penicillin and streptomycin was used as against 62.36 per cent when gentamicin, tylosin, lincomycin and spectinomycin was added (Kupferschmied *et al.*, 1991).

Penicillin and streptomycin the conventionally used antibiotics in semen extenders were found to be sensitive only to 27.1 and 20.34 per cent respectively of the isolates studied (Table 12, Fig.1). Penicillins at a concentration of 1000 IU/ml and streptomycin 1000 ug/ml of semen was not able to check the bacterial contamination of semen. Larger than this concentration was found to adversely affect sperm motility and metabolism (Rosza, 1950; Boryczko *et al.*, 1981). The ineffectiveness of this combination of antibiotics to eliminate the contaminants was observed by several workers (Lenz, 1956, Hirth *et al.*, 1967; Palli *et al.*, 1975; Kuradshiskii, 1980; Naidu, 1981; Rahman *et al*, 1983; Saikia *et al*, 1987; Shin *et al.*, 1988; Wayda, 1991). However Hardjoutomo (1990) reported that streptomycin and penicillins were very much effective against semen contaminants at the Lembang artificial insemination centre.

There is no available literature regarding the addition of antibiotics such as pefloxacin, doxycycline, ciprofloxacin and norfloxacin to semen extenders. In the light of the

present observations, studies are to be conducted regarding the right combination of these antibiotics and the effective concentration to be added. Sperm toxicity studies and fertility of the semen are to be thoroughly investigated before extensive usage of these antibiotics in frozen semen production.

Antifungal sensitivity of the isolates

Among the antifungal agents studied, nystatin was found to be the most effective. This was sensitive against 90.9 per cent of the fungal isolates tested. This result is in concordance with the observations made by Brown *et al* (1974) and Natalia (1987). The other agents studied were clotrimazole and amphotericin B which were sensitive to 81.8 per cent and 36.3 per cent of the fungal isolates.

Fungal agents are present in semen from all the centres under the present study. Natalia (1987) confirmed that nystatin at 50-100 units per millilitre could be added to diluted semen without affecting the viability or motility of bovine spermatozoa At present antifungal agents are not added to semen extenders in any of the centres in Kerala. Addition of nystatin to the semen extender is utmost important for the production of good quality frozen semen for insemination.

Summary

SUMMARY

The present study was conducted with the following objectives. 1. The assessment of the bacterial and fungal load of frozen bull semen produced in the three frozen semen production centres in Kerala, viz., Mannuthy (Centre A), Dhoni (Centre B) and Mattuppetty (Centre C). 2. Isolation and identification of bacteria and fungi present in frozen bull semen. 3. To study the antibiogram pattern of the isolates.

The mean bacterial load per dose of frozen semen in the three centres were 2260 ± 880.615, 825.91 ± 186.06 and 80.33 ± 18.47 respectively. The mean bacterial load per dose of semen differ significantly between these three centres ($P<0.05$). The mean values of Centre A was significantly greater than the other two centres.

The mean fungal load per dose of frozen semen in Centre A, B and C were 337.50 ± 88.447, 316.30 ± 41.055 and 15.0 ± 3.62 respectively. The mean fungal load of semen in Centre A and B were significantly greater than that produced in Centre C.

The mean coliform counts recorded per straw of semen in the three centres were zero, 7.273 ± 3.188 and 8.667 ± 4.43 respectively.

The pseudomonads count was detected in two media. In the media for pyocyanin the mean pseudomonad count per dose of semen in the centres were 527.00 ± 96.38, 651.82 ± 95.85 and 54.3 ± 19.04 respectively. The corresponding values in the media for flourescin were 725.00 ± 294.52, 596.80 ± 95.056 and 23.67 ± 907 respectively.

The mean non-lactose fermenters count recorded in the three centres were 5 ± 5, 5.545 ± 2.28 and 6.0 ± 3.094 respectively. The corresponding values for staphylococci and micrococci were 100 ± 5.774, 9.091 ± 4.463 and 2.33 ± 1.609 respectively.

Mycoplasma was not detected in any of the samples in Centre A, while four out of eleven samples from Centre B and two out of fifteen samples from Centre C were positive for Mycoplasma.

The total bacterial count exceeded 500 organisms per insemination dose in all the samples from Centre A and 72.7 per cent of samples from Centre B. None of the samples from Centre C was found to exceed 500 organisms per dose of semen.

The mean bacterial count per dose of semen from bulls below four years of age was significantly higher than those from bulls between the age of four and six years ($P<0.05$).

There was no significant difference between the fungal load in the semen of bulls in different age groups.

Similarly the mean bacterial and fungal load per dose of semen from crossbred Holstein Friesians were significantly greater than those of crossbred Jerseys ($P<0.05$).

The bacterial isolates from frozen semen samples from Centre A were *Bacillus licheniformis*, *B. cereus*, *Corynebacterium bovis*, *Staphylococcus aureus*, *Aerococcus viridans*, *Falvobacterium* sp., *Enterobacter aerogenes* and *Escherichea coli*. Seven from Centre B contained, *Bacillus alvei*, *B. brevis*, *B. cereus*, *Corynebacterium pseudodiphtheriticum*, *Staphylococcus epidermidis*, *S. aureus*, *alcaligenes faecalis*, *Flavobacterium* sp., *Pseudomonas alcaligenes*, *Pseudomonas mendocina*, *Edwardsiella tarda*, *Citrobacter koseri* and *E. coli*. The isolates from Centre C included *Corynebaterium hofmanii*, *Staphylococcus epidermidis*, *S. aureus*, *Micrococcus viridans*, *Kurthia* sp., *Alcaligenis* sp., *Pseudomonas alcaligens*, *Levinea* sp., *Enterobacter liquifacians*, *Edwardsiella tarda*, *Citrobacter koseri*, *Citrobacter intermedius*, *Citrobacter freundii*, *Providencia rettgeri* and *E. coli*.

Among the fungal isolates, *Candida albicans* and *Aspergillus niger* were identified from samples of Centre A, *Candida tropicalis*, *C. krusei*, *A. fumigatus*, *Microsporum*

gypseum and *Penicillum* sp. from Centre B and *C. pseudotropicalis* and *C. tropicalis* and *A. fumigatus* from Centre C.

A total of 59 bacterial isolates obtained from frozen semen were subjected to antibiotic sensitivity study. Amoxycillin, ampicillin, co-trimazine, cephalexin, cephaloridine, cloxacillin, erythromycin, furasolidone, gentamicin, nitrofurantoin, oxytetracycline, polymixin-B, triple sulpha, pefloxacillin, doxycycline, chloramphenicol, ciprofloxacin and norfloxacin were sensitive to 15.25, 10.17, 64.41, 57.63, 13.56, 22.03, 23.73, 22.03, 61.02, 32.20, 5.08, 72.88, 74.58, 94.91, 93.23, 93.23, 88.14 and 86.44 percentage respectively of the isolates tested. Penicillin and streptomycin the conventionally used antibiotics in semen extenders were sensitive to 27.1 per cent and 20.34 per cent respectively of the isolates tested.

The antifungal agents were also studied for its sensitivity for the fungal isolates. Nystatin was sensitive of 90.9 per cent, clotrimazole to 81.80 per cent and amphotericin B to 36.3 per cent of the fungal organisms.

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MICROBIAL LOAD IN FROZEN BULL SEMEN AND ANTIBIOGRAM OF THE ISOLATES

BY

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ABSTRACT OF A THESIS

Submitted in partial fulfilment of the
requirement for the degree

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1996

ABSTRACT

The mean bacterial load in the frozen semen procured from the three frozen semen production centres viz., Mannuthy (Centre A), Dhoni (Centre B) and Mattupetti (Centre C) were 2260 ± 880.65, 825.91 ± 186.06 and 80.33 ± 18.47 respectively. The mean bacterial load per dose of semen differed significantly between these three centres. The mean fungal load of semen in Centre A and B were significantly greater than that produced in Centre C. The mean coliform counts, pseudomonad count, staphylococci and micrococci count and mycoplasma count were detected in specific media. Mycoplasma sp. were not detected in Centre A while four out of eleven samples from Centre B and two out of fifteen samples from centre C contained Mycoplasma. The total bacterial load of all the samples from Centre A exceeded the ISI specification of 500 organisms per insemination dose of semen while 72.7 per cent of the samples from Centre B exceeded this limit. None of the samples from Centre C exceeded 500 organisms per dose of semen.

The mean bacterial load per dose of semen from bulls below four years of age was significantly higher than those from bulls between the age group of four and six years.

The mean bacterial and fungal load per dose of semen from crossbred Holstein Friesians were significantly greater than those of crossbred Jerseys.

The bacterial isolates from the semen from these centres where characterised. They belonged to the species, *Bacillus licheniformis*, *B. alvei*, *B. cereus*, *B. brevis*, *Corynebacterium bovis*, *C. pseudodiphtheriticum*, *C. hofmanii*, *Staphylococcus aureus*, *S. epidermidis*, *Aerococcus viridans*, *Micrococcus varians*, *Kurthia* sp. *Flavabacterium* sp., *Alcaligenes faecalis*, *Pseudomonas alcaligeness*, *Ps. mendocina*, *Levinea* sp., *Enterobacter aerogeness*, *Enterobacter liquifascians*, *Edwardsiella tarda*, *Citrobacter koseri*, *C. intermedius*, *C. freundii*, *Providencia rettgeri*, *Escherichia coli*.

The fungal isolates from semen, which were characterised belonged to the species *Candida albicans*, *C. pseudotropicalis*, *C. tropicalis* and *C. krusei*. The filamentous fungi isolated were *Aspergillus niger*, *A. fumigatus*, *Penicillum* sp. and *Microporum gypseum*.

Of the 20 antibiotics studied for the sensitivity pattern, pefloxacin, doxycycline, chloramphenicol, ciprofloxacin and norfloxacin were found to be sensitive to majority of isolates. Among the antifungal agents studied, nystatin followed by clotrimazole were sensitive to more

than 80 per cent of the fungal isolates. Nystatin may be used in semen extender for checking the fungal contaminants in frozen semen. The study recommends the addition of the new antibiotics to semen extenders after further field studies.

Appendices

Appendix A

1. Eosin Methylene blue Agar (Levine)

Ingredients	Grams/litre
Peptone	10
Dipotassium phosphate	2
Lactose	10 g
Agar	15 g
Eosin-Y	0.4 g
Methlene blue	0.065 g
pH at 25°C	7.1 ± 0.2

Procedure of preparation : Thirty seven grams of dehydrated culture medium was dissolved in one litre of distilled water, boiled and autoclaved.

2. Mac Conkey's Agar

Ingredients	Grams/litre
Pancreatic digest of gelatin	17
Pancreatic digest of casein	1.5
Lactose	10
Peptic digest of animal tissue	1.5
Bile salts	1.5
Sodium chloride	5.0

Agar	15 g
Neutral Red	0.03
Crystal violet	0.001
Final pH (at 25°C)	7.1 ± 0.2

Procedure of preparation : Fifty one and a half grams of the dehydrated culture medium was dissolved in one litre of distilled water, boiled and autoclaved.

3. **Mannitol salt Agar**

Ingredients	Grams/litre
Beef extract	1
Proteose peptone	10
Sodium chloride	75
D-Mannitol	10
Phenol red	0.025
Agar	15 g
Final pH at 25°C	7.1 ± 0.2

Procedure of preparation : One hundred and eleven grams of dehydrated culture medium was dissolved in one litre of distilled water, boiled and autoclaved.

4. PPLO Agar or Mycoplasma agar

PPLO Agar base

Ingredients	Grams/litre
Beef heart infusion from	250 gms
Peptone	10.0 gms
Sodium chloride	5.0 g
Agar	15 g
Final pH at 25°C	7.8 ± 0.2

Procedure of preparation : Thirty five grams of dehydrated basal medium were dissolved in one litre of distilled water, boiled and autoclaved. To every 70 ml of this autoclaved medium 20 ml of horse serum, 10 ml of yeast extract, one ml of 10 per cent Thalium acetate and one lakh units of penicillin G sodium were added aseptically.

5. Mueller Hinton Agar

Ingredients	Grams/litre
Beef infusion from	300
Casein hydrolysate	17.5
Soluble starch	1.5
Agar	15 g
Final pH at 25°C	7.4 ± 0.2

Procedure of preparation : Thirty eight grams of dehydrated culture medium was dissolved in one litre of distilled water, boiled and autoclaved.

6. **Potato dextrose Agar**

Ingredients	Grams/litre
Potato infusion from	300
Dextrose	20
Agar	15 g
Final pH at 25°C	5.6 ± 0.2

Procedure of preparation : Forty one grams of dehydrated culture medium was dissolved in one litre of distilled water, boiled and autoclaved.

7. **Pseudomonas Agar (For flourescein)**

Ingredients	Grams/litre
Tryptone	10.0
Proteose peptone	10.0
Dipotassium phosphate	1.5
Magnesium sulphate	1.5
Agar	15 g
Final pH at 25°C	70 ± 0.2

Procedure of preparation : 38.0 gms of the dehydrated culture medium was dissolved in one litre of distilled water. This was autoclaved.

8. **Pseudomonas Agar (for Pyocyanin)**

Ingredients	Grams/litre
Peptone	20
Magnesium chloride	1.4
Potassium sulphate	10.0
Agar	15 g
Final pH at 25°C	7.0 ± 0.2

Procedure of preparation : 46.4 gms of the dehydrated culture medium was dissolved in one litre of distilled water, boiled and autoclaved.

9. **Tryptic Soy Agar**

Ingredients	Grams/litre
Tryptone (Pancreatic digest of casein)	15.0 g
Soytone (Papaic digest of Soyabean meal)	5.0 g
Sodium chloride	5.0 g
Anhydrous glucose	1.0 g
distilled water	1000 ml
Final pH at 25°C	7.2 ± 0.2

Procedure of preparation - Autoclaved for 20 minutes at 121°C. 5% defibrinated calf blood was added just before pouring into plates at 45°C.

10. Dilution media (Buffered peptone Water)

Peptone	10.0 g
Sodium chloride	85.5 mM (5.0 g)
$Na_2HPO_4.12H_2O$	25.1 mM (9.0 g)
KH_2PO_4	11.0 mM (1.5 g)
Distilled water to	1000 ml

pH = 7.0 ± 0.2 (25°C)

This solution was placed in 10 ml screw - capped tubes each containing 9 ml of solution, then it was sterilised in the autoclave for 20 minutes at 121°C.

Appendix B

1. Amino acid decarboxylase test

Peptone	5 g
Meat extract	5 g
Glucose	0.5 g
Pyridoxal	5 mg
Bromocresol purple (1 in 500 solution)	5 ml
Cresol red (1 in 500 solution)	2.5 ml
Distilled water	1 litre

After dissolving the solids in water the pH was adjusted to 6.0. The amino acids ornithine and lysine was added at one per cent level to this basal medium after dividing it into three portions. The third portion was kept as control without adding amino acids.

The pH was readjusted to 6.0 and the media dispensed in one ml amounts in small tubes containing sterile liquid paraffin to provide a layer of five millimetre thick above the medium. This was autoclaved at 121°C for 15 minutes.

2. Christensens Urease test Agar

Peptone	1 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2 g

Phenol red (1 in 500 solution)	6 ml
Agar	20 g
Distilled water	1 litre
Glucose, 10 per cent solution	10 ml
Urea, 20 per cent solution	100 ml

The glucose and urea solutions were sterilized by filtration. The basal medium was adjusted to pH 6.8 and sterilized by autoclaving at 121°C for 30 minutes. After cooling to about 50°C, sterile solutions of glucose and urea were added and tubed as long slopes.

3. **Corn Meal Agar**

Ingredients	Grams/litre
Corn meal	50 gms
Agar	15 g
pH	6.0 ± 0.2

Procedure of preparation : 65 gms of the dehydrated culture medium was dissolved in one litre of distilled water, boiled and autoclaved.

4. **Kovac's reagent**

Amylalcohol	150 ml
p-Dimethyl-amino benzaldehyde	10 g
Conc. hydrochloric acid	50 ml

The aldehyde was dissolved in the alcohol and the acid slowly added. This was prepared and dispensed in small quantities and stored in the refrigerator.

5. **Malonate utilization and phenyl alanine deaminase test**

Medium

Ammonium sulphate	2.0 g
Dipotassium hydrogen phosphate	0.6 g
Potassium dihydrogen phosphate	0.4 g
Sodium chloride	2.0 g
Sodium malonate	3.0 g
DL-Phenyl alanine	2.0 g
Yeast extract	1.0 g
Distilled water	One litre

This medium was steamed for five minutes and filtered through filter paper. Five ml of a 0.5 per cent solution of bromothymol blue in absolute ethanol was added. This was distributed in 10 ml quantities and autoclaved at 121°C for 15 minutes.

6. **Nitrate reduction test**

Medium

Potassium nitrate (nitrite free)	0.2 g
Peptone	5 g
Distilled water	1 litre

The medium was tubed in 5 ml amounts and autoclaved at 121°C for 15 minutes.

Test reagents

Solution A - 8.0 g of sulphanitic acid dissolved in one litre of 5 N acetic acid.

Solution B - 5.0 g of naphthylamine dissolved in one litre of 5 N acetic acid.

7. Sabouraud's Dextrose Agar

Dextrose	40 g
Peptone	10 g
Agar	20 g
Distilled water	1000 ml
pH	5.6

The medium was sterilized in the autoclave at 120°C for 10 minutes.

8. Simmons Citrate Agar

Sodium chloride	5.0 g
Magnesium sulphate	0.2 g
Ammonium dihydrogen phosphate	1.0 g
Potassium dihydrogen phosphate	1.0 g
Sodium citrate	5.0 g
DL-Phenyl alanine	2.0 g

Agar	20 g
Bromothymol blue 0.2%	40 ml
Distilled water	1000 ml
pH	6.8

The medium was dispensed and sterilized by autoclaving at 121°C for 15 minutes and allowed to set as slopes.

9. Sugar fermentation test for identification of yeast

Fermentation media

Beef extract broth	3.0 g
Peptone	10.0 g
NaCl	5.0 g
Bromocresol stoch solution	1.0 ml
Distilled water	1000.0 ml

Ingredients were dissolved by heating and the pH was adjusted to 7.2. Grul broth was placed in 16 x 125 mm cotton plugged tubes with inverted Durham tube and sterilised by antoclaving at 121°C for 15 minutes.

10. Triple sugar iron medium

Polypeptone	20 g
Lactose	10 g
Sucrose	10 g
Dextrose	1 g

Sodium chloride	5 g
Ferrous ammonium sulphate	0.2 g
Sodium thiosulphate	0.2 g
Agar	13 g
Phenol red	0.025 g
Distilled water	1000 ml

The medium was sterilized at 115°C for 15 minutes, poured into tubes and cooled in a slanting position.