BACTERIAL LOAD IN THE NEAT, EXTENDED AND FROZEN BULL SEMEN AND ITS ANTIBIOGRAM

NAVYA, M.

DEPARTMENT OF VETERINARY GYNAECOLOGY AND OBSTETRICS

VETERINARY COLLEGE, BANGALORE

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR.

JUNE, 2012

BACTERIAL LOAD IN THE NEAT, EXTENDED AND FROZEN BULL SEMEN AND ITS ANTIBIOGRAM

Thesis submitted to the

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR

in partial fulfillment of the requirements

for the award of the degree of

Master of Veterinary Science

in

VETERINARY GYNAECOLOGY AND OBSTETRICS

By

NAVYA, M.

DEPARTMENT OF VETERINARY GYNAECOLOGY AND OBSTETRICS

VETERINARY COLLEGE, BANGALORE

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR.

JUNE, 2012

KARNATAKA VETERINARY, ANIMAL AND FISHERIES SCIENCES UNIVERSITY, BIDAR

DEPARTMENT OF VETERINARY GYNAECOLOGY AND OBSTETRICS

VETERINARY COLLEGE, HEBBAL, BANGALORE

CERTIFICATE

This is to certify that the thesis entitled ***"BACTERIAL LOAD OF NEAT, EXTENDED AND FROZEN BULL SEMEN AND ITS ANTIBIOGRAM"*** submitted by **Ms. NAVYA, M., I.D. No. MVHK 1027** in partial fulfillment of the requirements for the award of degree of **Master of Veterinary Science** in **Veterinary Gynaecology and Obstetrics** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

Bangalore

June, 2012

Dr. V. CHANDRASHEKARA MURTHY
Major advisor

Approved by:

Chairman : ______________________
Dr. V. CHANDRASHEKARA MURTHY

Members : 1. ______________________
Dr. A. KRISHNASWAMY

2. ______________________
Dr. SHRIKRISHNA ISLOOR

3. ______________________
Dr. T. SURYANARAYANA

4. ______________________
Dr. B. M. RAVINDRANATH

Affectionately Dedicated to

My Beloved Parents

And

My Friends

ACKNOWLEDGEMENT

Pages will not suffice to describe this pleasant moment of expressing gratitude to all those who have supported and helped me in completing this master degree programme

I cordially extend my thanks and gratitude to my mentor ***Dr. V. Chandrashekara Murthy,*** *Associate Professor, Dept. of Veterinary Gynaecology and Obstetrics, for his excellent supervision, unflagging support, stead-fast encouragement, immense patience, his painstaking help and enthusiasm for the work is a source for my motivation towards this work.*

With utmost respect, I avail this opportunity to express my deep sense of gratitude and whole hearted thanks to ***Dr. A. Krishnaswamy,*** *Professor and Head, Dept. of Veterinary Gynaecology and Obstetrics, for his good nature, valuable suggestions, and support at all the stages of my master degree programme as a member of my advisory committee. I confess that it has been a great fortune and proud privilege for me to be associated with him during my master degree programme.*

I feel much honor to express my deep sense of gratitude to my advisory committee members ***Dr. Shrikrishna Isloor****, Associate Professor, Dept. of Veterinary Microbiology, Veterinary College, Bangalore,* ***Dr. T. Suryanarayana****, Assistant Professor, Dept. of Veterinary Medicine, Veterinary College, Bangalore and* ***Dr. B. M. Ravindranath****, Assistant Professor and Head, Teaching Veterinary Clinical Service Complex for their sustained encouragement, technical guidance, timely suggestions and interest in solving problems related to my research work and support as members of my advisory committee.*

I express my earnest and profound sense of indebtedness to ***Dr. G. Sudha*** *Assistant Professor, Dept. of Veterinary Gynaecology and Obstetrics and* ***Dr. T.G. Honnappa****, Associate Professor, Dept. of Veterinary Gynaecology and Obstetrics for their constructive suggestions, motivation, critical review and meticulous corrections in my thesis and support at all the stages of my master degree programme that enabled to complete my research work.*

I extend my thanks to ***Dr. Mahesh,*** *Additional Director of farms, Nandini Sperm Station, Bangalore, for his help during the research work, providing the* *laboratory facilities and working environment which were of great help during the research programme. I consider it would be incomplete without extending my heartfelt thanks and profound sense of gratitude to all the staff of Nandini Sperm Station not only for their help in various capacities but also for their modesty and integrity. I would also extend my heartfelt thanks to the managing director of KMF for granting permission to carry out my research in the NSS.*

I wish to express my hearty thanks to my seniors ***Chethan, Mahesh, Aditi, Kavyashree, Stanzin, Babu, Murthy, C N A, Vinodh, Anand Patil*** *and* ***Narasimha Murthy sir*** *for their suggestions and from whom I have always got help from one way or other. I also acknowledge my colleagues* ***Vibha****,* ***Shwetha, Aswartha, Kavya, Dost*** *and* ***Hakim*** *for providing good working environment during my stay in the department and also to my junior colleagues* ***Sowmya, Chitra, Prashanth, Ashok, Yatish*** *and* ***Amaregowda*** *for their help, support and for making my stay comfortable and memorable during the entire period of my study. I also thank* ***Puttu****,* ***Rajanna, Siddaram, Satish kumar*** *and other supporting staff of the Dept. for their help and support.*

Words fail to express the magnitude of support, inspiration, joyous atmosphere, generous cooperation and inspiring moral support extended during the course of my study given by my friends the ***V10****team,* ***Thanu, Vibha,*** *and* ***Viji*** *and for making memorable moments for life. I deeply express my sincere thanks to all my friends, whose continuous inspiration, encouragement and affection, boosted up my morale during the period of study.*

I am meticulously thankful and grateful to my beloved parents, elder brother, aunt, uncle, cousins and all my family members whose hands were always raised for my well being and success.

Bangalore,

June, 2012 **(Navya, M.)**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

I. INTRODUCTION

Indian dairying has emerged, as the world's largest producer of milk and this was possible to a greater extent with the introduction of artificial and frozen semen technology. India has the largest infrastructure in the world with 64 frozen semen bull stations and more than 60,000 artificial insemination centres which are producing about 36 million frozen semen straws annually and inadequacies have led to the substandard semen quality and poor conception rates (National project for cattle and buffalo breeding-NPCBB, 2006).

The minimum standard protocol (MSP) for semen production is not followed strictly in most parts of the country. Also, there is lack of legislative monitoring for standardization and certification of semen stations. Quality of semen varies from state to state, and among semen stations within the state. So, it is necessary to follow minimum standard protocol for better quality semen production in frozen semen station and it is imperative that more thrust should be given to semen research and production in bulls. The Office Internationale Epizooticus (OIE) has laid down certain norms and protocols for frozen semen production and quality control for international shipment and one such norm is with regard to the bacterial load in frozen semen. The standard for acceptable colony forming units is 500 CFU per ml of frozen semen. Even under careful and strictly monitored conditions, semen often gets contaminated during collection, processing and preservation. The microorganisms can affect the male reproductive function directly, causing the agglutination of motile sperms, reducing the ability of acrosome reaction and causing alterations in cell morphology and indirectly, through the production of reactive oxygen species generated by the inflammatory response to the infection (Moretti *et al*., 2009).

Several studies both in India (Singh *et al*., 1990 and Ramaswamy *et al*., 2002) and abroad (Wayda, 1991) have established that the most of frozen semen isolates are resistant to commonly used antibiotics namely Penicillin and Streptomycin. In view of the ever growing multi- drug resistance and introduction of new generation antibiotics for reducing the bacterial load in semen, drug sensitivity of the bacterial isolates from semen becomes imperative and mandatory (Sandeep Jaisal *et al*., 2000). Examination of semen characteristics, such as concentration, progressive motility and viability are routine procedures for assessing semen quality in AI centres. However, studies in domestic animals of these semen characteristics were often non significantly correlated with fertility. Computer assisted semen analysis (CASA) system has been used to measure the proportion of sperm exhibiting motility and motility characters. Live and dead sperm count in frozen semen samples is often misleading. Thus, the ideal test for measuring the quality of semen should be simple, user-friendly, easy to interpret and inexpensive. One such test could be the evaluation of microbial load in the semen and Methylene blue reduction test. The production of frozen semen of a larger dimension needs a quality control service to maintain supply of good quality frozen semen and to attain a good quality of frozen semen microbial analysis should be carried out at each and every step of processing.

Keeping the above factors in view, the present study was designed with the following objectives:

* To record the load of viable bacteria in the neat, extended and frozen semen of HF bulls.
* To estimate the viable bacterial load of the extender used during semen processing.
* To evaluate the antimicrobial sensitivity and resistance pattern of the microbes in the frozen semen samples.
* To compare the viable bacterial load with the age of the bulls if any.
* To correlate the viable bacterial load with the Methylene blue reduction time if any.

Review of Literature

II. REVIEW OF LITERATURE

2.1 Total bacterial load in the semen

Semen can be infected with many microorganisms which retain their viability for long intervals at freezing temperatures, the use of cryopreserved germplasm for artificial insemination and in other related new reproductive technologies, are of concern to the health authorities. According to the latest survey (1988), more than 260 million doses of bull semen were produced worldwide, out of which 95% were deep frozen, and 20 million were moved internationally (Thibier, 2002).

The importance of the bacterial numbers in semen initiated the research work on bacteriology of semen as early as in 1944 by Gunsalus and co-workers. The bacterial numbers in semen varies from place to place and among the semen production centers.

Highly contaminated semen is a characteristic for stallion's ejaculate (Madsen and Christensen, 1995) and even sanitary measures like washing cannot avoid contamination.

Semen collection in farm animal species is not a sterile procedure, and some degree of contamination with bacteria cannot be avoided (Clement *et al.*, 1995; Varner *et al.*, 1998; Althouse and Lu, 2005; Aurich and Spergser, 2007 and Bielanski, 2007).

Specific microorganisms gain access to the semen as a result of viremia or bacteraemia. On the other hand, many organisms are present due to local infections in parts of the genital tract or can be associated with blood cells due to trauma or with inflammation and or trauma of the urinary tract and the preputial cavity. As a result, presence of infection is often an intermittent process, with pulses of high concentration of pathogen in the semen, followed by low concentrations or none at all (Thibier and Guerin, 2000).

2.1.1 Microbialload in neat semen:

Naidu *et al.* (1982) reported from Tirupathi (AP, India) that the bacterial count in neat semen of jersey bulls varied from 3.666×10^3 to 41.277×10^3 per ml.

Studies by Fache *et al*. (1985) have shown that the level of saprophytic flora can be as high as 4 to 5 logs CFU/ml in freshly collected semen.

Marinov (1988) reported the bacterial count ranging from 200000 to 2 million per ml of seminal fluid.

Ajit Kumar *et al.* (1994) reported that mean bacterial load in the first ejaculate was 1100 per ml as against 4860 organisms per ml in the second ejaculate of crossbred bulls from Thrissur, Kerala.

Sandeep Jaisal *et al.* (2000a) reported that the bovine bulls (33.3%) and buffalo bulls (24.1%) carried the bacterial load from 10,000 to 50,000/ml respectively. On an average bacterial count per ml of neat semen ranged from 5000 to 56,000 in 46 ejaculates of bovine bulls and 4100 to 18,000 in 29 ejaculates of buffalo bulls.

Studies by Jesus Luis Yaniz *et al.* (2010) indicate that ejaculated semen from commercial rams can frequently contain bacterial flora, in concentrations of up to 10^8CFU/ml.

2.1.2 Microbial load in frozen semen of bulls:

Bartlett *et al*. (1981) and Kupferschmied *et al.* (1991 a and b) stated that under practical conditions elimination of microbial contamination completely from frozen semen was not practicable and some contamination with non pathogenic microorganisms was unavoidable and its importance should not be over estimated.

Contamination of preserved semen with microbes poses a great threat to the success of AI programme. Even under careful conditions, semen often gets contaminated during collection, processing and preservation (Naidu *et al*., 1982). Frozen semen, which is being used now in AI, cannot be expected to be free from microbes. However, in spite of hygienic status of the semen and precautions employed during processing, microbes gain entry in to the semen (Mohanty *et al*., 1988 and Ramaswamy *et al.*, 1997).

A host of microbes belonging to bacterial species, Mycoplasma, fungi and yeast types have been reported to be present in frozen semen by many researchers (Naidu *et al*., 1982; Gangadhar *et al*., 1986 a and b; Bindra *et al*., 1994; Ajit Kumar *et al*., 1994; Ramaswamy *et al*., 2002 and Sandeep Jaisal, 2000a). The magnitude of the infection potential of one bull whose semen is used in AI is very large and affects fertility of large number of females. Indian standards institute (ISI, 1976) has established a standard of 500 non- pathogenic bacteria per dose of frozen semen straw, which should also be free from pathogenic bacteria (Naik *et al*., 1990).

Bacterial contaminants in semen survive at -196°C in liquid nitrogen and acquire a certain level of resistance to antibiotics (Ronald and Prabhakar, 2001) and accounts for the contamination of approximately 50% of frozen semen samples (Wierzbowski *et al.*, 1984).

Gangadhar *et al*. (1986 a;1986 b) reported that mean bacterial counts per ml of frozen buffalo bull semen at three centres in Tirupathi, south India, were 442± 100, 10559± 3059 and 61± 14.

Singh *et al.* (1990) reported that the mean bacterial load in chilled and frozen bull semen at different hours and days of preservation with or without antibiotics and the figures per ml of chilled semen were (12.05± 2.041, 13.24± 2.24, 14.22± 2.02 and 15.70± 1.92) x 10^3 at 0, 24, 48 and 72 hours of preservation, respectively on the other hand, figures per ml of frozen bull semen were (11.56± 1.67, 5.84± 0.19, 4.51± 0.29, 3.32± 0.18 and 2.5 ±0.16) X10^3 before freezing, immediately after freezing, 30days, 60 days and 90 days of freezing, respectively.

The range of microbial load in frozen buffalo semen per ml varied between 70000 and 540000 (Ramaswamy *et al*., 1990), 0 and 23000 (Naik *et al*., 1990), 200 and 1820 (Prabhakar *et al*., 1993) organisms and for frozen bovine semen were 120 and 16800 (Mohanty *et al.*, 1988), 300 and 2 lakh (Ramaswamy *et al*., 1994) and 0.81X10^3 to 39.04X10^3 (Rathnamma *et al*., 1997).

In the study conducted by Shukla (2005) the bacterial load of frozen semen were found to be lower (7.00±0.91; 79.00±0.76; 60.00±0.87 bacteria per ml) due to prepucial wash of the bulls at 12 hr and 20 minutes before semen collection and maintenance of strict aseptic conditions at the time of semen collection.

According to the study conducted by Abro *et al*. (2009) out of hundred samples of frozen semen of cattle examined, 7 were found positive for various bacterial isolates, while 93 were negative without any bacterial growth.

2.1.3. Standards regarding Microbial load in frozen semen:

According to the Indian Standards Institution (ISI, 1976) frozen semen should be free from pathogenic bacteria and should not contain more than 500 non pathogenic bacteria per insemination dose.

Wierzbowski (1981) reported that the limit of 500 organisms per ml of frozen semen as recommended by Ostaszko (1976) to International Organization of biological Standards was not accepted.

A review of the statutory requirements laid down in different countries regarding frozen semen meant for domestic use or for import or export purposes reveals that no rigid standards with regard to bacterial numbers exist in United states of America (Bartlett, 1984), United Kingdom, Switzerland (Kupferschmied, 1991 a & b), Sweden and Australia. Further, in these countries the accent is on the production of semen with minimal contamination by observing maximum hygienic precautions in different stages of production and processing of semen, without attaching undue importance to bacterial counts in semen.

2.2 Relationship between the microbial load of semen and the age of bulls:

A comparison of the plate counts of the semen samples from bulls of various ages was made by Almquist *et al*. (1949). The lowest numbers of bacteria were found in semen from the bulls in the 6 -9 year old age groups, while the greatest numbers of bacteria were found in semen from the oldest group(>10 years) of bulls attributed to the deeper epithelial crypts in the prepuce and penis of the older bulls. Since differences in plate counts of semen between age groups were not marked, there appears to be no important relationship between the age of bulls and the average plate count of semen.

2.4 Microbial contamination during semen collection, processing and storage:

Water used for buffer preparation during semen extension should be micro filtered to make the water pyrogen free. Pyrogens in distilled water may cause clumping of spermatozoa (Roberts, 1971).

The saprophytic flora of the prepuce in healthy semen donors comprises numerous bacterial species which may become associated with the semen at ejaculation and during collection. Under appropriate environmental conditions some of the bacteria may behave as opportunistic pathogens and represent a significant risk to inseminated females (Wierzbowski, 1981).

The freezing of semen enables many infectious agents to survive and cryoprotectants may render antibiotics less effective (Bartlett, 1981).

Low temperatures were found to reduce the antimicrobial activity of some antibiotics (Nickolai *et al*., 1985).

Recently developed commercial semen extenders (e.g. Biociphos-plus, from IMV) free of egg yolk and milk, eliminated the potential risk of contamination of semen doses with bacteria and Mycoplasma from substances of animal origin (Bousseau *et al*., 1998).

Lindeberg *et al*. (1999) and Aurich and Spergser (2007) reported about the unavoidability of collecting contaminated semen using a closed AV during the semen collection.

There are no available data provided by manufacturers on the microbial load of recently produced LN. Nevertheless, based on limited observation, it can be assumed that the level of contamination of LN is low and limited to ubiquitous microorganisms. Morris, (1999) referred to less than 100 colony forming units of aerobes per 10 kg of LN. From a practical point of view, complete sterilization and maintenance of sterility in a robust system like LN production system, might be a very demanding task. Accordingly, some ubiquitous bacterial agents can be expected in any commercially produced LN.

Due to the interference of glycerol with the antimicrobial properties of the antibiotics, it has been recommended to add the antibiotics directly to the raw ejaculates rather than to the extender with the cryoprotectant (Bielanski *et al*., 2000).

Retrospective studies about LN cryotanks revealed bacterial and fungal contaminants in the LN detritus and the identified bacteria during the study were ubiquitous environmental microorganisms and rare opportunistic pathogens of low significance in producing disease in humans and animals (Bielanski *et al*., 2003).

Ingredients (milk, blood serum or serum albumin, sucrose, sorbitol, and other sugars; cryoprotectants like DMSO) used in semen extension may act as stabilizers for microorganisms at freezing temperatures. Many microorganisms have been found to tolerate very high DMSO concentrations without visible toxic effects and some (*Acinetobacter spp*., *Corynebacterium spp*., *Bacillus spp*., *Streptomyces spp*.,) are even capable of multiplication in a growth medium containing 2% to 45% DMSO (Hubalek, 2003).

A broad range of microorganisms have been isolated from long term, cryopreserved, commercial bull semen in extenders containing antibiotics, as well as from both nitrogen tank detritus and the liquid nitrogen phase (Bielanski *et al*., 2003).

Considering that there are no methods to sterilize large quantities of LN, all cryotanks used for storage of biological samples should be considered potentially contaminated with at least environmental microorganisms. Most germplasm are stored in large capacity cryotanks, which may create a potential cross-contamination of clean samples in case of breaking or leaking of infected samples into the LN. Over the storage time, due to the exposure of cryotanks to the laboratory environment during refilling and handling of specimens, ice crystals will form on the walls of the vessels and aggregated ice and sediment may trap bacteria, fungal spores and debris, posing a risk of microbial transmission to stored samples (Morris, 2003 and 2005). In contrast, a recent study suggests that long-term banking of germplasm in the LN phase, even over 35 years is a safe technique for preservation of genetic materials with a rather low potential risk of cross-contamination when the specimens are properly sealed (Bielanski *et al.*, 2003).

Keeping in view the current international trends in disease control, it is possible that extenders having ingredients of animal origin (egg yolk) can be the source of microbes / bacteria, consequently resulting in the contamination of semen (Bousseau *et al*., 1998).

It was estimated that more than half of the pathogens in the laboratory/ andrology clean rooms originated from the normal flora of people working at these locations and the majority of them were opportunistic pathogens (Cobo and Concha, 2007).

Dilution of the ejaculates with sterile diluents will further decrease the concentration of contaminants (Bielanski, 2007).

During semen collection it is difficult to avoid contamination with the saprophytic bacteria of the prepuce or with bacteria from the environment, as evidenced in different species (Althouse *et al.,* 2000; Aurich and Spergser, 2007 and Akhter *et al*., 2008).

A recent study in rams with suspected infertility, the most common isolates included *E. coli*, *Staphylococci*, and *Proteus* species which were recovered from seminal samples with normal and reduced quality, which suggested that they were probably either commensals of the reproductive tract, or contaminants (Otter, 2008).

Although ejaculated semen tends to be handled under the most sterile conditions, sperm collection and processing of commercial straws of semen is usually carried out in a non-sterile environment and with non-sterile equipment which may produce undesirable collateral effects on the sperm characteristics. Additionally, uncontrolled diseases present in the animals may also be transmitted to offspring by using infected semen samples (Givens and Marley, 2008).

Most microorganisms in the form of “clean” cultures or in association with germplasm can survive storage at low temperatures, including in liquid nitrogen (-196^{0}C) (Tedeschi and De Paoli, 2011).

A quantified risk assessment for cross-contamination of cryopreserved livestock semen in LN has not been evaluated as of yet. It can be speculated, therefore, that the risk of cross-contamination between semen stored for commercial purpose in LN may be negligible as compared with experimental semen samples where the worst case scenario was created by a very high titre of microbial agents introduced into the cryosystem (Pomeroy *et al*., 2010).

2.5 Influence of microbial load on quality of the semen:

A large number of microorganisms have been isolated from semen and the prepuce. Twenty seven different types of bacteria, fungi and blastomycetes have been identified in 337 semen samples in a study conducted during the year 1984 and almost identical flora in 139 preputial washing liquids by Flastscher and Holzmann, which means that there is controversy concerning the true effects of such agents on freezing, fertilising power and the appearance of inflammatory processes (Parez, 1984).

Damage to the midpiece of the spermatozoa was positively correlated with the number of bacteria (Danek *et al.,* 1996). Whereas Diemer *et al*. (1996) reported a deleterious effect of bacterial contamination on sperm function, altering the structure of sperm and affecting the motility.

The spermicidal effect of the bacteria is concentration dependant and is an established fact (Auroux *et al*., 1991; Diemer *et al*., 1996).

The bacterial contaminants of semen have been a major concern for most semen production laboratories as it adversely affects the semen quality (Diemer *et al*., 1996) and subsequent fertility (Ochsendrof and Fuch, 1993; Aitken, 1995 and Griveau *et al*., 1995). Macrophages and polymorphonuclear granulocytes, which form the first line of defense against microorganisms, produce reactive oxygen species (ROS) to kill these microorganisms. ROS is released outside the cells and may react with the spermatozoa in their vicinity (Ochsendrof, 1998).

Different strategies may be employed to minimize the effects of bacterial contamination on extended semen and as the bacterial concentration is below a threshold level, fertility is not affected (Althouse *et al*., 2000).

A non significant negative correlation was found between sperm motility and the bacterial load (-0.228) (Ahmed and Greesh Mohan, 2001) and 0.27 and -0.29 respectively by Ahmed and Greesh Mohan (2002).

Although, the microorganisms present may be non- pathogenic, the accumulation of their metabolic products are known to reduce the sperm motility (Ronceanu *et al*., 1973 and Ronald and Prabhakar, 1999) and microbial contamination in the germ line can impact semen quality, also affecting the DNA molecule.

The presence of microorganisms, especially the bacteria in the ejaculates can affect fertilization directly, by adhering to spermatozoa (Diemer *et al*., 1996), impairing their motility (Kaur *et al*., 1986) and inducing acrosome reaction (El- Mulla *et al*., 1996). Microbes can also have an indirect effect by producing toxins (Morrell, 2006).

There is increasing evidence that toxic soluble factors released by bacteria, such as α-haemolysin, Shiga- like toxin, lipopolysaccharides and peptidoglycan fragments may also have a deleterious effect on spermatozoa (Schulz *et al*., 2010). Presence of bacteria in semen samples can adversely affect the DNA integrity of sperm and this effect seemed to be dependent upon the initial bacterial load and bacterial growth rate, since progressively more sperm showed DNA fragmentation as the bacterial population increases after in vitro incubation.

2.6 Effect of antibiotics on bacterial sensitivity, viability and fertility of spermatozoa

Foote and Salisbury (1948) and Almquist *et al*. (1949) first proposed that bacterial contaminants in bovine semen could be controlled by adding antibiotics during semen extension.

The addition of penicillin and streptomycin to frozen semen reduced the motility of sperms on thawing (Stoyanov, 1987). Further, he also quoted that the best antibiotic for semen was gentamicin and the conception rate of cows inseminated with semen containing gentamicin was 15% higher than that containing conventional antibiotics (penicillin + streptomycin).

Effects of non- conventional antibiotics in frozen semen on fertility have been sparsely assessed in cattle (Ahmed and Foote, 1985 and 1986; Lorton *et al*., 1988b; Kupferschmied *et al*., 1991a and b; Kommisrud *et al*., 1996).

Shin *et al*. (1988) and Hasan *et al.* (2001) have indicated that the antibiotics (GTLS) have a broader spectrum of microbial control in frozen bovine semen than Strepto-penicillin. Thus, the presence of effective antibiotics in semen extender significantly reduces the concentration of bacterial metabolites and increases the available energy for spermatozoa (Ala-ud-Din *et al.,* 1990), resulting in better semen quality/ fertility (Lorton *et al*., 1988b).

Shin *et al.* (1988) reported that treatment of raw semen with GTLS for 3-5 min at 35°C followed by dilution with an extender containing these antibiotics effectively controlled *Mycoplasma, Ureaplasma spp*., *Haemophilus* and *Camphylobacter*. The antibiotics were not detrimental to seminal quality (Lorton *et al.,* 1988a; Krause *et al*., 1989 and Hasan *et al*., 2001) or viability (Ericsson *et al*., 1990) and the method has been adopted as a standard treatment for bull semen processed for commercial AI in the developed countries - CSS® (1993).

The mean bacterial load after thawing with conventional antibiotics namely Penicillin + Streptomycin and Chloramphenicol + Gentamicin was 5380 ± 0.16 and 273 ± 0.042, respectively (Singh *et al*., 1990).

Resistance of bull frozen semen isolates to either one or combination of the following antibiotics namely Penicillin, Colistin, Tetracycline, streptomycin and Polymixin-B and sensitivity to Gentamicin, Kanamycin, Chloramphenicol, Enrofloxacin, Lincomycin and Neomycin was recorded by Rahman *et al*. (1988), Ahmed *et al*. (1989), Singh *et al.* (1990), Ramaswamy *et al*. (1990), Rakesh Sharma *et al*. (1994) and Sandeep Jaisal *et al*. (2000b).

Several studies both in India (Singh *et al.,* 1990 and Ramaswamy *et al*., 2002) and abroad (Wayda, 1991) have established that most frozen semen isolates are resistant to commonly used antibiotics namely, Penicillin and Streptomycin. In view of the ever growing problem of multi drug resistance, and introduction of new generation antibiotics for reducing the bacterial load in semen, drug sensitivity of the isolates is mandatory (Sandeep Jaisal *et al*., 2000b).

Control of bacterial growth is usually performed by the use of semen extenders containing antibiotics with broad-spectrum bactericidal or bacteriostatic activity (Maxwell and Salamon, 1993 and 2000).

Current international standards with regard to the antibiotic components of semen extenders have made it necessary to look for alternatives for the Strepto-penicillin containing extender (CSS®, 1993 and 2002 and Hasan *et al*., 2001).

Presence of effective antibiotics in semen extender significantly reduces the concentration of bacterial metabolites and increases the available energy for spermatozoa (Din *et al*., 1990; Tanyildizi and Bozkurt., 2003), resulting in better semen quality/ fertility (Lorton *et al.,* 1988a).

Keeping in view the quality and shipment of frozen semen across the continent, microbial sterility of it is mandatory. Thus, several new extenders such as Triladyl, Biociphos, which have come into vogue for freezing semen in bovines which contain antibiotics such as Lincomycin and Spectinomycin in order to control the spread of Mycoplasma and ureaplasma that are the obligatory pathogens in frozen semen samples.

Many studies have revealed that most of the isolates being sensitive to Amikacin, Chloramphenicol, Gentamicin, Kanamycin, Norfloxacin and Tobramycin (Singh *et al*., 1990; Ramaswamy *et al*., 1990; Bindra *et al*., 1994; Sandeep Jaisal., 2000b; Ahmed and Greesh Mohan., 2001; Ronald and Prabhakar., 2001b and Ahmed and Greesh Mohan., 2002). Further, incorporation of Chloramphenicol, Gentamicin, Amikacin and Norfloxacin lowered the bacterial load to almost negligible levels in the frozen semen (Ahmed *et al*., 1989; Singh *et al*., 1990; Gupta and Maurya, 1993; Sandeep Jaisal, 2000b; Ahmed and Greesh Mohan, 2001; Ronald and Prabhakar, 2001b and Ahmed and Greesh Mohan, 2002).

Conventionally benzyl penicillin and streptomycin sulfate alone or in combination of a 1000IU/ml and 1000mg/ml respectively are used during semen extension (Hussain *et al*., 1990; Ali *et al*., 1994; Sansone *et al*., 2000; Andrabi *et al*., 2001 and Akhter *et al.*, 2008).

Systemic studies of the relatively new antibiotic combination (GTLS) have revealed that it is not detrimental to post –thaw semen quality or fertility in bovine (Gerard *et al*., 1995; Bousseau *et al.*, 1998; Hasan *et al.*, 2001 and Andrabi *et al*., 2001).

Ramaswamy *et al*. (1997) reported that potential pathogenic microbes are able to survive in the processing, freezing and storage conditions, though the frozen semen contains antibiotics to control them. There is great potential to spread diseases through artificial insemination caused by multi-resistant bacteria.

Antibiotics in the semen extenders cannot completely suppress or eliminate contamination in the collected and diluted semen and even freezing can be survived by some species of bacteria (Varner *et al.*, 1998 and Pickett and Voss, 1999).

Antibiotic sensitivity conducted for the mixed cultures revealed that Amikacin and Chloramphenicol were highly effective and did not vary significantly on comparison (Prabhakar *et al*., 1993).

In mixed cultures the percentage of sensitivity for Chloramphenicol, gentamicin, Streptomycin and Penicillin-G were 81.6%, 55.0%, 18.5% and 0% respectively (Ronald and Prabhakar, 2001).

Andrabi *et al*. (2001) concluded that the combination of Gentamicin, Tylosin and Lincospectin was found better for the improvement of fertility of frozen buffalo and Sahiwal bull semen and the fertility rates for Strepto-penicillin based frozen semen of buffalo bull were 41.66% and 55.2% for GTLS- containing frozen semen respectively while, the fertility rates were significantly higher ($P<0.0001$) for GTLS- based frozen semen of Sahiwal bull (78.78%) than SP- containing frozen semen (69.6%) of the same species. In contrast, Kommisrud *et al.* (1996) reported a little higher non- return rate with Strepto-penicillin containing semen extender as compared to GTLS based extender in cattle.

Lowered fertility rates in buffaloes reported by Andrabi *et al*. (2001) with Strepto-penicillin as compared to GTLS based AI doses could be due to occurrence of pathogenic strains of bacteria particularly Pseudomonas in buffalo bull semen resistant to Strepto-penicillin (Aleem *et al*., 1990 and Hasan *et al.*, 2001).

The reduction in motility when gentamicin was used in a study conducted by Ahmed *et al.* (2002) was attributed to the toxic effects of Gentamicin (Jasko, 1993). Lower motility in the extender containing penicillin + streptomycin and no antibiotics might be due to higher level of bacterial load (Bindra, 1991).

Ahmed and Greesh Mohan (2002) reported that the bacterial load of semen containing Gentamicin, Norfloxacin and Amikacin did not exceed 500 organisms per straw and the variation in mean bacterial load was significant (P>0.01) between the bulls and the treatment.

The organisms were highly sensitive to Ampicillin (86.4%), Gentamicin (77.9%) and Chloramphenicol (76.27%) and with respect to Penicillin and Streptomycin it was 22.0 and 0 percent respectively (Gupta and Maurya, 1993). While, Ahmed and Greesh Mohan (2001) reported that Amikacin (89.71%), Norfloxacin (88.24%) and Gentamicin (85.29%) were most effective antibiotics and the routinely used penicillin and Streptomycin were effective against 67.65% and 23.53 per cent of isolates, respectively. Ramaswamy *et al. (*2002) observed the sensitivity to Chloramphenicol, Ciprofloxacin, Gentamicin and Neomycin as 100% and for Streptomycin as 86.2% and for both Amoxicillin and penicillin- G as 0%.

Addition of antibiotics to semen extender was one of the major advances to significantly improve the fertility potential of artificial insemination in bovines (de Jarnette *et al*., 2004).

In a study on effect of antibiotics on the structure of spermatozoa, Gentamicin was found to affect the sperm membrane, acrosomal integrity, morphological abnormalities of the head, mid-piece and tail and also associated with reduced motility of the sperms (Akhter *et al*., 2008).

To control bacteria load in semen samples, addition of antimicrobials are commonly practiced in semen extenders and one problem associated could be the antimicrobial resistance of the bacteria. Hence, prudent use of antimicrobials in the semen diluents is an essential component to the overall control of contaminant bacteria in extended semen (Althouse *et al*., 2008).

2.7 Methylene blue reduction test and its relation to quality of semen:

Studies on the rate of glycolysis or on the rate of respiration provided a sound basis for developing a quick, easy test for determining the quality of semen. A method for determining the reaction rate in the metabolism of spermatozoa was proposed (Beck and Salisbury, 1943) which consisted of determining the time in minutes, required for semen diluted at a standard rate with yolk- citrate, to reduce a 1: 40,000 concentration of Methylene blue. The Methylene blue reduction was correlated with spermatozoa numbers and initial motility of the spermatozoa. However, the usual numbers of bacteria was found not to interfere with the results of test on fresh semen samples (Gunsalus *et al*., 1944).

Methylene blue reduction time measures the metabolic activity of sperm cell. Many scientists have used this test for assessing bull semen quality (Sergin, 1940 and Sorenson, 1942). Under aerobic conditions the rate of hydrogen transfer at the metabolism of sperm cell may be determined from the rate at which Methylene blue is reduced (Bhosrekar, 1990).

Erb *et al*. (1950) and Bishop *et al.* (1954) suggested that the reduction time was directly related to motility and sperm concentration and inversely to the percentage of dead spermatozoa. Semakov, (1963) established a strong correlation between dehydrogenase activity of semen, sperm motility and concentration of spermatozoa. Upsenskli, (1967) established significant correlation between MBRT and fertility. Good quality semen reduced the Methylene blue within 3 to 6 minutes and the semen which retained the colour for 9 minutes or longer were not suitable for artificial insemination purposes (Bhosrekar, 1990). Bishop *et al*. (1954) recorded an average MBRT of 7.5 minutes when the semen was diluted in egg yolk phosphate buffer saline, 3.3 minutes in extender with insufficient fructose, 6.6 minutes in presence of adequate levels of fructose in the extender.

Materials and Methods

III. MATERIALS AND METHODS

The present study was undertaken at the semen collection centre – Nandini Sperm Station (a unit of Karnataka Milk Federation), Kakolu, Hessaraghatta lake post, Bangalore. The semen station is situated at 921metres above the mean sea level at a longitude of 77.35° and latitude of 12.58° north. The study was conducted from March to April during the year 2012.

3.1Animals

Six adult healthy Holstein Friesian bulls aged between two to six years, stationed at Nandini sperm station, Kakolu, Bangalore were used for this study. All the bulls were housed in individual pens with facilities for abundant water supply and maintained under uniform environmental and managemental conditions. They were offered concentrate feed (17% DCP and 70% TDN) at the rate of one per cent body weight and green and dry fodder (Maize, Lucerne) was adjusted to meet the NRC recommended requirements. Semen from all the bulls were collected at weekly intervals using artificial vagina. Immediately after collection, semen was evaluated for various macro and microscopic characteristics according to Salisbury *et al.* (1978). The subjective sperm motility of each ejaculate was evaluated in a phase contrast microscope equipped with a warm stage (38°C Leitz, Germany) and its sperm concentration was determined in a photometer (Bovine Photometer, L'Aiglon, IMV India Ltd.). The ejaculates were extended in routinely used Tris egg yolk fructose citric acid glycerol extenders (TEYG) processed at room temperature (20-22°C) and subsequently frozen by programmed freezing method.

3.2Post thaw semen evaluation

The semen samples were thawed in water bath according to standard procedure for bull semen straws in French mini straws. The straws were thawed at 37°C for 30 seconds and emptied in tubes for further evaluation.

3.3Motion kinetic parameters using computer assisted analyzer

Computer assisted sperm motility analysis (CASA) was performed in a CASA system (IVOS S/N 6057/ 10.9I, Hamilton Thorne Research Inc., USA.) immediately after neat semen collection and post thaw, maintaining the sample at room temperature (20-22 °C).

3.4 Preparation of glasswares

The glassware used in the study were prepared by soaking them in detergent (Teepol) solution over night. The following day, they were washed thoroughly in running tap water, and then rinsed in deionised / distilled water (DW). The oven dried glassware were packed and sterilized in hot air oven for one hour at 160 C as per Collee *et al*. (1996).

3.5 Media reagents and chemicals

The media and reagents were either obtained from Hi-media, Mumbai or prepared in the laboratory as per the standard procedures. Known quantity of dehydrated media was suspended in distilled water, boiled to dissolve and then sterilized by autoclaving at 121^{o}C for 15 min at 15 psi.

3.6 Bacterial load of semen

3.6.1 Bacterial load of neat semen

The neat semen samples of six consecutive collections of the six HF bulls were collected and evaluated in the laboratory of the semen bank at Nandini sperm station.

The bacterial load was estimated by pour plate method (Cruikshank *et al.*, 1975). The semen samples were either inoculated directly in to the commercially available Standard plate count agar (Hi- media Lab, Bombay) or after serial dilution if the count exceeded 300 CFU/ plate. The counts were made after 48hr of incubation at 37°C using a Quebec colony counter. The bacterial load was expressed as CFU per dose of semen (per ml of neat semen).

3.6.2 Bacterial load of extender and extended semen

The semen samples of six consecutive collections of the six HF bulls were extended using the Tris egg yolk fructose citric acid glycerol (TEYG) extender and the extended samples were collected for the estimation of microbial load of the extended semen samples. The microbial load was estimated by pour plate method. Also samples of the extender prepared were collected and subjected for evaluation of microbial load.

3.6.3 Bacterial load of frozen semen

The frozen semen samples of six consecutive collections of the six HF bulls under present study were collected for the microbial analysis.

The microbial load was estimated by pour plate method (Cruikshank *et al*., 1975). The semen samples were inoculated directly in to the commercially available Standard plate count agar. The counts were made after 48hr of incubation at 37°C and the bacterial load expressed as CFU per dose (0.25 ml) of semen.

3.7 Antibiogram

Semen samples were cultured directly in nutrient broth and incubated at 37°C overnight. A sterile swab dipped in this culture was spread over sterile Mueller – Hinton agar petriplates and in vitro antibiogram was carried out as per standard single disk diffusion method (Bauer *et al.*, 1966) using bio- discs (Hi- media, Mumbai, India)and the following antibiotic test discs were laid on the dried inoculums with the help of a dispenser. The zone of inhibition was estimated using antibiotic zone measuring scale in mm.

Table 1. Details of antibiotics used for antibiogram profile

S. No:	Antibiotics	Code	Concentration
1	Gentamicin	GEN	10mcg / disc
2	Tylosin	TL	15mcg/disc
3	Lincomycin	L	10mcg/disc
4	Spectinomycin	SPT	100mcg/disc
5	Enrofloxacin	EX	10mcg / disc
6	Amoxyclav	AMC	30mcg/disc

3.8 Methylene Blue Reduction Time (MBRT)

The neat semen samples were subjected to Methylene blue reduction test. In a clean test tube of 10 ml capacity, 1ml of extended semen and 0.1ml of Methylene blue (1:40,000) solution was added and incubated in water bath at 45°C and the time required for the complete discolouration of Methylene blue was recorded in minutes.

3.9 Statistical analysis

The characteristics of HF bulls were generated for microbial load of neat, extended and frozen semen samples. The data generated was tabulated and subjected to one-way analysis of variance to examine the variation between the bulls for the trait. The data pertaining to the MBRT and the bacterial load was subjected to correlation tests (Pearson) and that of the antibiotic sensitivity test to chi-square test.

Results

IV. RESULTS

The results of the present study to assess the total bacterial load of bull semen are presented below.

4.1 Bacterial load in the neat semen

A total of 34 semen samples obtained from 6 bulls were analysed for total bacterial load of neat semen. The mean bacterial load per ml of neat semen of HF bulls under study was 3200 ± 7.4 CFU/ml.

The mean values of total viable bacterial load (per ml) of neat semen recorded for six HF bulls in the present study respectively were 1700 ± 4.1; 4800 ± 12 ; 3300 ± 7.5 ; 1100 ± 3.8 ; 1900± 6.0 and 7500 ± 56 CFU/ml. The mean bacterial load in the neat semen of six bulls in six different collections under present study ranged from 200 to 30000 CFU per ml. The statistical analysis revealed significant variations in the total bacterial load among bulls ($P<0.05$).

4.2 Bacterial load in the extended semen

The mean bacterial load per ml of extended semen of HF bulls under study was 230 ± 0.18 CFU/ml.

The mean viable bacterial load of the extended semen for six HF bulls were 150 ± 0.67; 220 ± 0.6; 170 ± 0.42; 240 ± 0.93; 370 ± 0.56 and 240 ± 0.51 CFU per ml respectively. The mean bacterial load in the extended semen of six bulls (36 samples) in six different collections ranged from 200 to 500 CFU per ml. The variation in the mean values among the bulls for the bacterial load of extended semen were found to be non-significant ($P<0.05$).

4.3 Bacterial load in the frozen semen:

The mean bacterial load per dose of semen recorded in the frozen semen of the HF bulls under study was 260 ± 0.82 CFU/dose. The mean bacterial load per dose of semen recorded in the frozen semen of the six HF bulls(36 samples) under the study were 450 ± 2.3; 180 ±0.65; 83± 0.31; 160 ± 0.24; 550± 2.4 and 140 ±0.75 CFU per dose respectively.

The minimum and maximum values obtained for total bacterial loads per insemination dose (per straw) were 100 to 1600 CFU per dose. The total bacterial counts exceeded 500 organisms per insemination dose (per straw) in 11.76% of the samples examined while, in 82.35% of the samples the bacterial counts remained below the specifications of 500 organisms per insemination dose. In 0.05% of the samples the bacterial count was found to be 500 organisms per insemination dose.

The variation in the mean values among the bulls for the bacterial load of frozen semen were found to be highly significant ($P<0.05$).

4.4 Growth of bacteria in neat, extended and frozen semen samples

In the present study, none of the neat semen samples were negative for bacterial growth. While in extended semen samples, five out of the 36 (13.88%) samples were negative for bacterial growth/ sterile. Out of the 34 frozen thawed semen samples, 29 samples (85.29%) showed growth of bacterial colonies, whereas 7 samples (20.58%) were sterile.

4.5 Significance between bacterial load of neat, extended and frozen semen

Critical differences showed that the mean values of total viable bacterial load were significantly ($P<0.05$) different between the bacterial load of neat and extended semen and also between the bacterial load of neat semen and frozen semen whereas, no significance was found between the mean values of total viable bacterial load of extended semen and frozen semen samples.

4.6 Correlation of bacterial load with Methylene Blue reduction test.

The average Methylene blue reduction time for the six bulls under the present study observed were 2.75, 5.41, 3.16, 6.33, 5.00 and 4.66 minutes respectively. The correlation of Methylene blue reduction time with the bacterial load of neat semen was found to be negative ($r = -0.40$) and the relationship was non-significant.

4.7 Comparison of bacterial load of semen of two different age groups.

The mean bacterial load per ml of neat, extended and frozen semen of HF bulls aged below 4 years and bulls aged above 6 years under the present study did not vary and was 2600 ± 5.20 CFU/ml; 260 ± 0.11CFU/ml; 290 ± 1.6 CFU/ml respectively.

Critical differences showed that the mean values of total viable bacterial load for the bulls aged below 4 years and above 6 years were significantly ($P<0.05$) different between the bacterial load of neat and extended semen and also between the bacterial load of neat semen and frozen semen whereas, no significance was found between the mean values of total viable bacterial load of extended semen and frozen semen samples.

4.8 Antibiogram

The *in vivo* drug sensitivity pattern of 36 ejaculates revealed resistance to one or more antibiotics. The highest sensitivity (86.10%) was observed with Lincomycin followed by Spectinomycin (81.94%), Tylosine (79.16%), Gentamicin (76.38%), Amoxyclav (38.88%), and Enrofloxacin (27.77%). On the other hand, the highest per cent of resistance was observed to Enrofloxacin (72.22%), followed by Amoxyclav (61.11%), Gentamicin (23.60%), Tylosine (20.83%), Spectinomycin (18.05%) and Lincomycin (13.88%). The maximum resistance was against Enrofloxacin (72.22%) and the minimum was against Lincomycin (13.88%).The sensitivity of Strepto-penicillin used in the semen extender was found to be 50%.

Table 2. Mean bacterial load of neat semen samples of bulls:

Bull No.	Mean ± SE of bacterial load of neat semen (Cfu/ml)
480	1700 ± 4.10
483	4800 ± 12.00
484	3300 ± 7.50
545	1100 ± 3.80
547	1900 ± 6.00
548	7500 ± 56.00

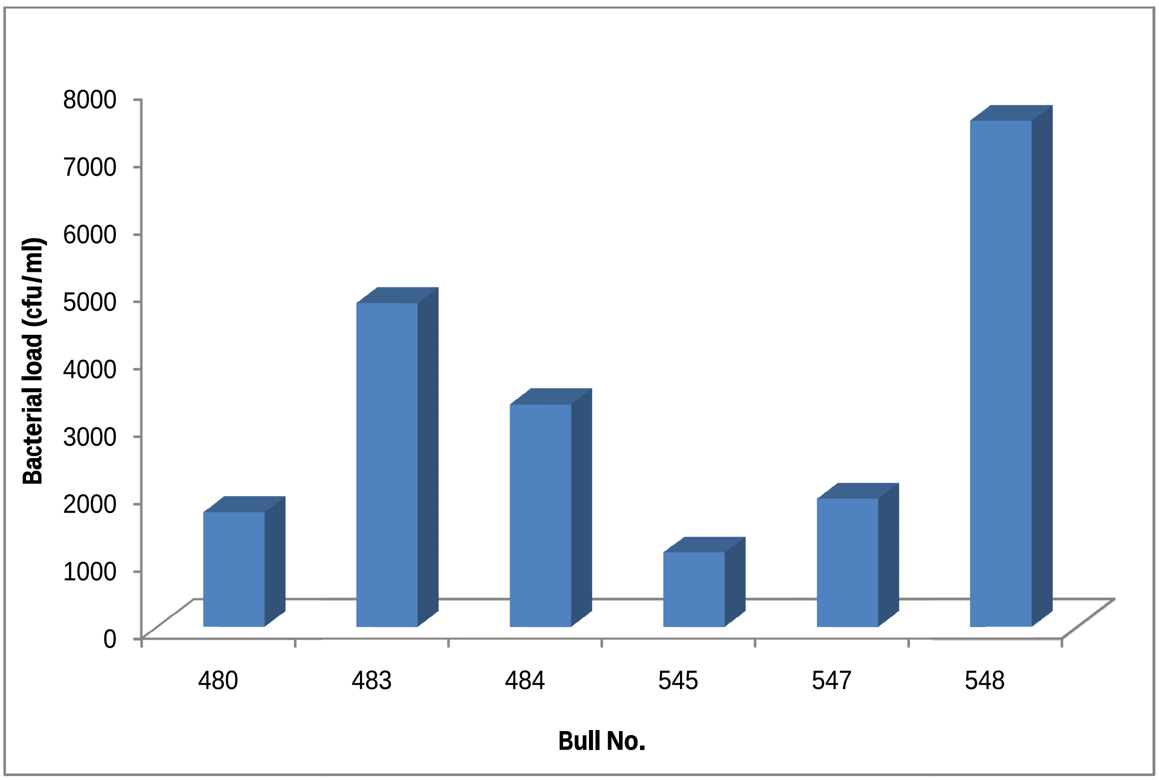

Fig.1. Mean bacterial load of neat semen samples.

Table 3. Mean bacterial load of extended semen samples of bulls:

Bull No.	Mean ± SE of bacterial load of extended semen (Cfu/ml)
480	150± 0.67
483	220 ±0.60
484	170± 0.42
545	240 ±0.93
547	370 ±0.56
548	240 ±0.51

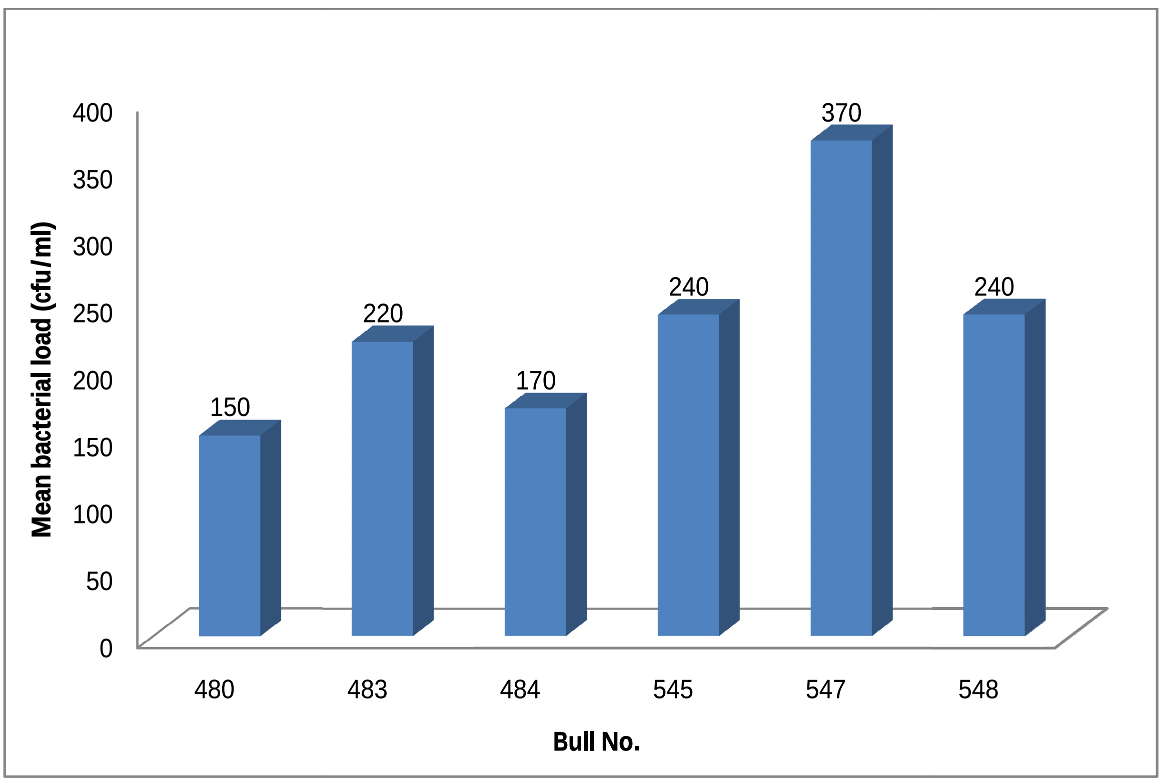

Fig. 2. Mean bacterial load of extended semen samples.

Table 4. Mean bacterial load of frozen bull semen samples:

Bull No.	Mean ± SE of bacterial load of frozen semen(Cfu/ml)
480	450 ± 2.30
483	180 ± 0.65
484	83 ± 0.31
545	169 ± 0.24
547	550 ± 2.40
548	140 ± 0.75

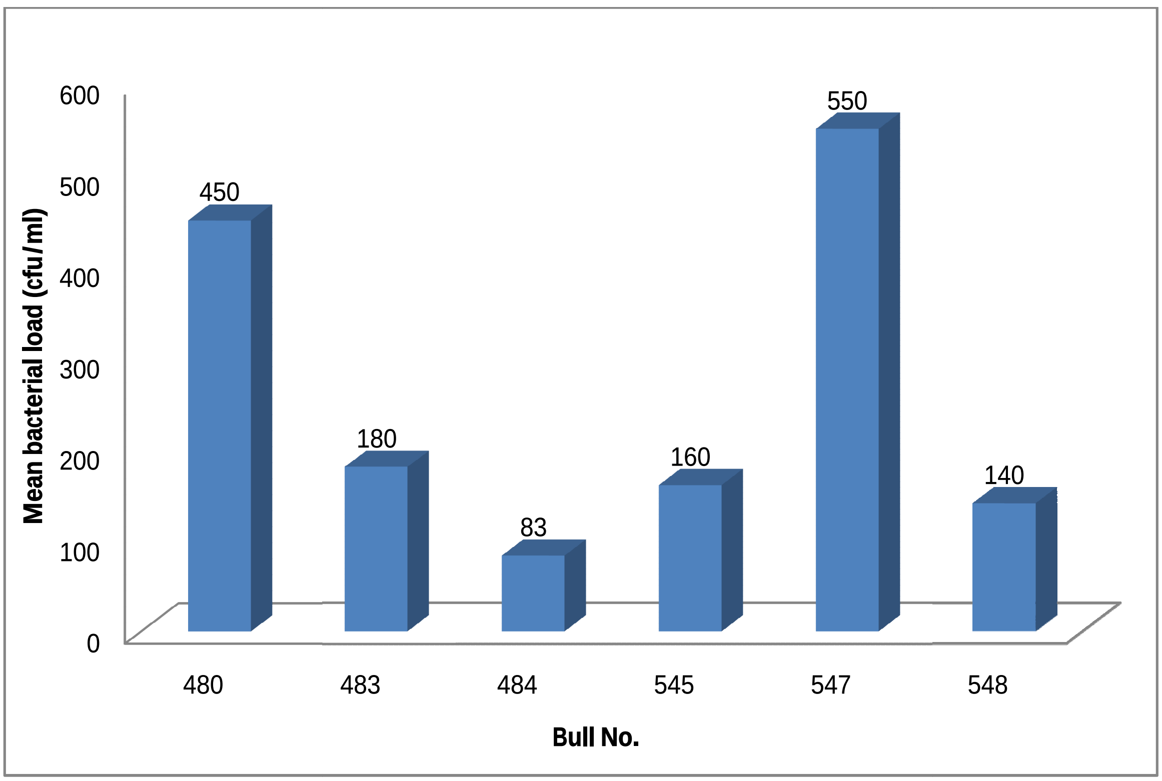

Fig. 3. Mean bacterial load of frozen semen samples.

Table 5. Overall Mean bacterial load of neat, extended and frozen bull semen samples

Mean ± SE of Bacterial load of Neat, Extended and Frozen semen.			
Parameter	**Neat semen**	**Extended semen**	**Frozen semen**
COLONY FORMING UNITS	**3200 ± 740**	**230 ± 18**	**260 ± 82**

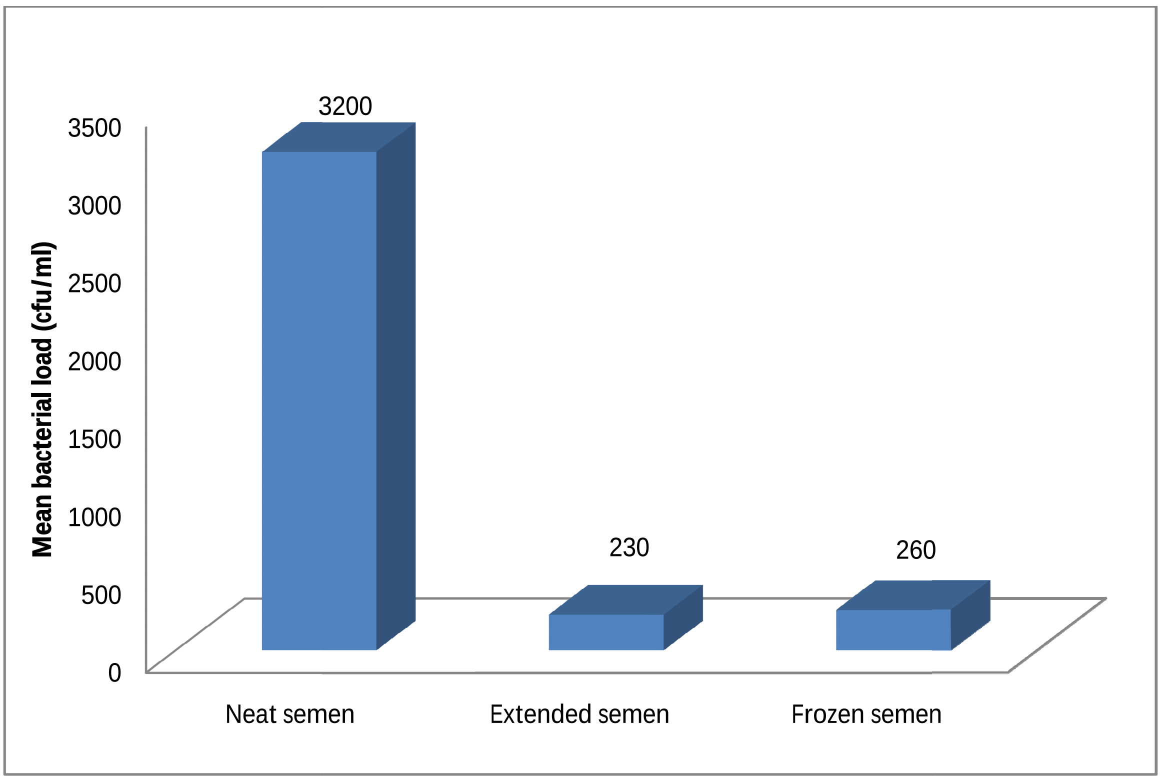

Fig. 4. Mean bacterial load of neat, extended and frozen semen samples.

Table 6. Sperm concentration and Methylene Blue Reduction Time:

Bull No.	Average concentration of spermatozoa (x 10^6 /ml)	Methylene Blue Reduction Time (in minutes)
480	1467	2.75
483	990.16	5.41
484	1679.5	3.16
545	945.16	6.33
547	1029.83	5
548	1434.75	4.66

Table 7. Methylene Blue Reduction Time and mean bacterial load of neat semen:

Bull No.	Mean Bacterial load of neat semen (Cfu/ml)	Methylene Blue Reduction Time (average in minutes)
480	1700 ± 4.10	2.75
483	4800 ± 12.00	5.41
484	3300 ± 7.50	3.16
545	1100 ± 3.80	6.33
547	1900 ± 6.00	5
548	7500 ± 56.00	4.66

Table 8. Relationship of bacterial load of neat semen with MBRT:

Relationship of bacterial load of neat semen with MBRT	
Parameter	**Methylene Blue Reduction Time**
Bacterial load	**-0.4**

Table 9. Sensitivity and resistance patterns of bacterial cultures of semen samples to various antibiotics

Antibiotic	Sensitive		Resistant	
	No.	Percent	No.	Percent
Gentamicin	26	76.47%	8	23.52%
Tylosine	27	79.41%	7	20.58%
Lincomycin	29	85.29%	5	14.70%
Spectinomycin	28	82.35%	6	17.64%
Enrofloxacin	9	26.47%	25	73.52%
Amoxyclav	13	38.23%	21	61.76%

Note: No. – Number of samples tested.

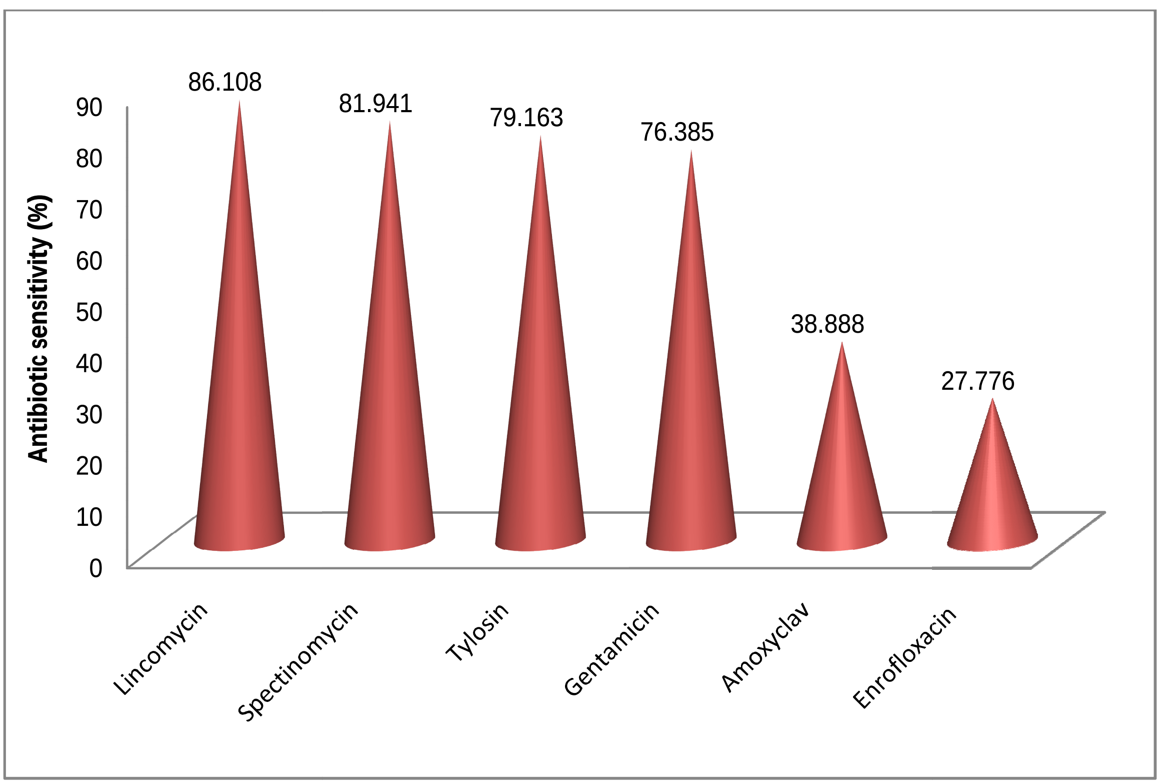

Fig. 5. Antibiotic sensitivity patterns of frozen semen samples.

Discussion

V. DISCUSSION

The spermiogram of a bull is the key characteristic and it should be optimum in assessing the fertility of that bull. Fertility of a bull is solely dependent on the qualitative characters such as colour, mass activity, individual motility, pH, morphology, fructolysis index, MBRT and RRT and quantitative characters such as volume and concentration of spermatozoa of the semen produced. In addition, bacterial load of semen constitutes an equally important component. Contamination of the semen with bacteria can happen at various stages of processing and handling. In view of this and the need for effective monitoring of semen processing it is felt important to evaluate the bacterial load. This approach enables to check the contamination points and to recommend appropriate measures to overcome the problems associated with such contamination. With the very same purpose, the present study was undertaken to estimate the bacterial load of semen at various stages of semen processing, their antibiogram profiles and suitable recommendations.

Bacterial load of neat semen

The presence of high bacterial load, in semen causes lethal effect on the spermatozoa due to their toxins and metabolic end products (Bindra *et al.*, 1994) and also leads to rapid decline in sperm motility (Naidu *et al.*, 1982). In the present study, semen samples from six HF bulls in the age group of 2-6 years were collected at weekly intervals following strict hygienic precautions. Estimation of the bacterial load in the aforementioned semen samples revealed a significant variation between the mean values of bacterial load per ml of neat semen ($P<0.05$). The values ranged from 1100 ± 3.80 to 7500 ± 56 cfu/ml and the mean bacterial load of neat semen was 3200 ± 7.4 cfu per ml.

As for the earlier reports on estimation of bacterial load in the bull semen samples, the values observed in the present study is in conformity with the findings of Ajit Kumar *et al.* (1994) and Hasan *et al*. (2001) who reported the bacterial load of neat semen samples to be 1100 to 4860 cfu/ml and 234 to 3280 cfu/ml respectively.

The mean bacterial load of neat semen recorded in the present study was much lower when compared to the high values of 2,00,000 cfu/ml (Almquist *et al.,* 1949), 6,300 cfu/ml (Kher and Dholakia, 1984), 60 to 9600 cfu/ml (Batish *et al.,* 1982), 0.2 to 20,00,000 cfu per ml of seminal fluid (Marinov, 1988), 3,46,235.25 ± 342.47cfu (Prabhakar *et al.*, 2001), 10,000 to 50,000 cfu/ ml (Sandeep Jaisal *et al.*, 2000) in bovine semen.

The variations reported by different scientists and the observations made in the present study might be ascribed to the organisms harboured in the genital tract of the bull, surface contamination, individual and breed variations and geo-climatic/environmental conditions.

Bacterial load of extender and extended semen:

In the present study, the mean bacterial load was estimated in the extender as well as extended semen to ascertain the sterility of extender and then to check the quality of semen extended. The findings indicated that mean bacterial load in the extended semen varied between150.00 ± 0.67 to 370.00 ±0.56 cfu/ml, with the mean bacterial load of

230.00 ± 0.18 cfu per ml. Further, statistical analysis, showed that the difference in the mean bacterial counts between the bulls was non-significant ($P<0.05$). The mean viable bacterial load per ml in the extended semen recorded in the bulls of the present study was found to be lower than 500 cfu/ml. However, some samples showed no bacterial growth. The mean bacterial load in the extended semen observed in the present study was much lower when compared to high load at 1,24,440.83 ± 194.24cfu per ml of extended semen (Prabhakar *et al.,* 1993), 3694.17 ± 1974.03cfu per ml of extended semen (Ahmed and Greesh Mohan., 2002).

The observations made in the present study is in accordance with the findings of Fache *et al.* (1985) who also reported viable bacterial load lower than 500 cfu/ml, and the reduction of bacterial load in extended semen samples recorded in the present study may be ascribed to quantitative effects of dilution, hygienic precautions followed during preparation of extenders and the process of extension and addition of antibiotics.

Bacterial load of frozen semen

The frozen semen technology has become popular since 1780 (earliest documented use of AI) in view of the long term preservation of semen, to distribute advantageous genes and eliminate the problem of venereal diseases. This could be a double edged sword as usage of contaminated semen especially with the microbial agents associated with venereal disease. Further, the freezing of semen enables many infectious agents to survive and cryoprotectants may render antibiotics less effective (Bartlett, 1981). Hence, the situation demands, great caution to ensure that the same vehicle does not spread genetic defects or pathogens. Even though, with the advantage of bulls to donate up to 1000 doses from a single ejaculate, the potential for the spread of diseases through contaminated semen is considerable.

In the present study, a significant variation was observed for the mean values of bacterial load per ml of frozen semen between the bulls ($P<0.05$) on statistical analysis and it varied between 83.00 ± 0.31 to 550.00 ± 2.4 cfu/ml, with a mean bacterial load of 260.00 ± 0.82 cfu per ml. In the present study, about 82.35% of the frozen semen samples showed less than 500 cfu/ dose, as per the standards mentioned by the Bureau of Indian Standards.

The overall mean of bacterial load recorded in the present study is in close conformity to that of 190 to 426 cfu/ml (Hasan *et al.,* 2001). On the other hand, the mean bacterial load was much lower compared to the values in frozen semen recorded by various authors viz., 2922.22 ± 164.27 (Rakesh Sharma *et al*., 1994), 3301.67 ± 323.18 (Bindra *et al*., 1994), 18334.13 ± 103.02 (Ronald and Prabhakar., 2001) and 5097 ± 1133 (Mohanty *et al.,* 1988), 5380 ± 16 (Singh *et al.,* 1990) and 2244.17 ± 960.18 (Ahmed and Greesh Mohan., 2002) in bovine semen.

The mean range of microbial load observed in the present study was also much lower when compared to the values of 7 x 10^3 to 540 x10^3 (Ramaswamy *et al.,* 1990), 0 to 23x 10^3 (Naik *et al*., 1990), 0. 2 x 10^3 to 1.82 x 10^3cfu/ml (Prabhakar *et al*., 1993), 0.12x 10^3 to 16.8x 10^3 (Mohanty *et al*., 1988), 0.3 x 10^3 to 20 x 10^3(Ramaswamy *et al*., 1994) and 0.81 x 10^3 to 39.04 x 10^3 (Rathnamma *et al.,* 1997) in bovine frozen semen. Several workers (Batish *et al.,* 1982 and Gangadhar *et al.,* 1986a) reported the contribution of empty straws, sealing powder in the semen straws to the degree of contamination in the frozen semen samples. The wide variation reported by different workers may also be attributed to different antimicrobial treatments given to the prepuce for washings, technique of collection and storage of samples, technique used for isolation and counting of bacteria and individual bull and age (Bindra *et al.,* 1994 and Rakesh Sharma *et al*.,1994), from laboratory contamination during semen processing for cryopreservation, liquid nitrogen used for freezing and time of storage of frozen semen (Salle,1974 and Bielanski *et al.,* 2003).

In contrast, the mean bacterial load of frozen semen recorded in the present study was higher compared to that reported by Shukla, 2005 (48.60 ± 0.73 bacteria per ml). In the study of Shukla, (2005) and such low counts is attributed to prepucial wash of the bulls before semen collection and maintenance of strict aseptic conditions at the time of semen collection and processing.

From the above results, it can be concluded that constant microbial monitoring is essential for maintaining the hygienic status of semen and to make it safe for artificial insemination (Rathnamma *et al.,* 1997) and critical control points monitoring should be made mandatory for all semen processing laboratories.

Methylene Blue Reduction Test:

Methylene blue reduction test is routinely used as a qualitative test to assess the metabolic activity of the spermatozoa. The test is recommended for routine prediction of semen quality (Van demark *et al*., 1944) considering its simplicity, reliability, practical applicability and cost effectiveness. The time taken for reduction of Methylene blue was shown to be correlated with spermatozoa counts and initial motility of the spermatozoa, as the reduction time is directly related to the motility and the concentration of the spermatozoa in the semen sample. However, it is not a precise measure of the ability of spermatozoa to survive storage as might be desired (Beck and Salisbury., 1943). Hence in the present study, the neat semen samples were subjected to MBRT and the average Methylene blue reduction time observed for the six semen samples collected from bull no.1 to 6 were 2.75, 5.41, 3.16, 6.33, 5.00 and 4.66 minutes respectively. As for the concept of MBRT is concerned, it works on the principle of viability of either spermatozoa or bacteria or both. Considering this fact in view, the average MBRT values of aforementioned neat semen samples from 6 bulls could be influenced by the load of both spermatozoa as well as bacteria. However, in this study, it unambiguously appeared that the bacterial load did not significantly affect the variation of MBRT among the samples, since there was clear reciprocative relationship between sperm concentration and MBRT (Table 6). However, such inverse relationship was not evident in case of bacterial load vis-a-vis semen samples (Table 7). The correlation of Methylene blue reduction time with the bacterial load of neat semen was found to be negative (r = - 0.40) and the relationship was non-significant. The findings of this study is supported by Gunsalus *et al.* (1944) who reported that the bacterial load of the semen samples did not interfere with the results of Methylene blue reduction test .

Relationship between bacterial load of neat semen and the age of bulls:

In all, 6 bulls belonging to 2 different age groups (3 bulls of 2 years and 3 bulls of 4-6 years) were identified, their semen samples collected and tested. Bulls in the age group of 2 years showed 3220 ± 126.8 cfu / ml of viable bacteria where as those of 4-6 years had 2550 ± 524.7 cfu/ml of bacterial load. In a similar study, Almquist *et al*. (1949) reported that lowest number of bacteria (84 x 10^3 to 240 x 10^3cfu/ml) were found in semen from bulls in the aging upto 9 years, while greatest numbers (400x10^3cfu/ml) of bacteria were recorded in semen from the oldest group of bulls (above 10 years of age) and the finding/ variation was attributed to high bacterial count in semen from a single bull. Findings of our study clearly indicated that the age factor of the selected bulls (< 6 years) did not influence the bacterial load which is in accordance with Almquist *et al.* (1949). Generally, for optimal semen production purposes, the bulls up to 6-8 years are maintained in the semen collection centres, as per the minimum standard protocols and Government of India.

Antibiogram:

Antibiotics are routinely added to the extenders before semen extension to check the bacterial contamination, if any. However, in spite of addition of antibiotics, there have been reports of elevated cfu levels of bacteria in the frozen semen than that after dilution (Hasan *et al.*, 2001). This has been attributed to the antimicrobial resistance in turn due to it's indiscriminate usage. In view of such ever-growing multi-drug resistance, introduction of new generation antibiotics for reducing the bacterial load in semen, drug sensitivity of the bacterial isolates of semen is imperative (Sandeep Jaisal *et al*., 2000b).Current international standards with regard to the antibiotic components of semen extenders have made it necessary to look for alternatives for the Strepto-penicillin containing extender (CSS, 1993; Hasan *et al*., 2001).

The *in vitro* antibiogram of the semen samples of the bulls carried out in this study is presented in Table 9.The results revealed that Lincomycin is the most effective drug (86.10%), followed by Spectinomycin (81.94%), Tylosine (79.16%), Gentamicin (76.38%), Amoxyclav (38.88%) and Enrofloxacin (27.77%). The conventional antibiotic Strepto-penicillin was only 50% sensitive as observed in the present study.

On the other hand the high per cent of resistance to Strepto-penicillin (50%) observed in the present study corroborate with those of Rahman *et al*. (1983), Singh *et al.* (1990) and Ramaswamy *et al.* (1990).

Bacteria may develop resistance either following the continuous use of a drug for a long time or due to injudicious usage. Penicillin-G and Streptomycin have been extensively used for semen extension, which may have led to the development of a high degree of resistance. On the contrary, Gentamicin, Tylosine, Lincomycin, Spectinomycin are either new antibiotics or not have been used in extending semen and thus have a higher efficiency.

The results of the present study showed that the percentage of resistance to Gentamicin (23.6%) was lower and the observation is in agreement with reports of Sandeep Jaisal *et al*. 2000b. A higher resistance to penicillin and Streptomycin was also observed by Singh *et al.(*1990), Ramaswamy *et al.* (1990), Gupta and Maurya (1993), Prabhakar *et al.* (1993), Ramaswamy *et al*. (1994), Bindra *et al*. (1994), Sandeep Jaisal (2000b), Ahmed and Greesh Mohan (2001), Ronald and Prabhakar (2001) and Ahmed and Greesh Mohan, (2002). Based on antibiogram, a number of studies have demonstrated the value of incorporating Gentamicin in extending semen.

The addition of Gentamicin reduced the bacterial load to negligible levels as demonstrated by Singh *et al*. (1990), Sandeep Jaisal (2000b), Ahmed and Greesh Mohan (2002).Salisbury *et al*. (1978) reported that gentamicin was non-toxic to the spermatozoa at a dose level of 1000µg per ml of extender, and at a similar dose used by Singh *et al*. (1990) in their studies found it to be useful in reducing the bacterial load. Panda *et al*. (1990) reports that there is no significant difference in the percentage of livability and motility without any adverse effect on the quality of semen. Ahmed *et al*. (2001) reported that the percentage of progressively motile sperms was higher in semen containing Gentamicin or Norfloxacin. Further, the combination of Gentamicin, Tylosine and Lincospectin (GTLS) is demonstrated to be more effective for controlling microorganisms including *Mycoplasmas*, *Ureaplasmas*, *Campylobacter fetus*, *Haemophilus somnus* and *Pseudomonas* in bovine semen than the standard combination of Strepto-penicillin (Shin *et al.*, 1988; Hasan *et al*., 2001) and have indicated that the antibiotics (GTLS) have a broader spectrum of microbial control in frozen bovine semen than Strepto-penicillin. Andrabi *et al*. (2001), Akhter *et al*. (2008) and Ala-ud-Din *et al.* (1990) found better conception rates from frozen semen containing GTLS antibiotics. Total aerobic bacterial counts were found to be significantly lower in semen samples treated with GTLS than those of Strepto-penicillin as reported by Aleem *et al*. (1990).

As per the guidelines stated by the OIE in the terrestrial animal health code (article 4.6.7.) 2011, a mixture of antibiotics should be included with a bactericidal activity at least equivalent to that of the following mixtures in each ml of frozen semen: Gentamicin (250µg), Tylosine (50µg), Lincomycin-spectinomycin (150 / 300µg); penicillin (500 IU), streptomycin (500µg), Lincomycin-spectinomycin (150 / 300µg);

Amikacin (75 µg), divekacin (25µg) and the names of the antibiotics added and their concentration should be stated in the *international veterinary certificate*.

It may be concluded that bacterial contamination in frozen semen can be minimised by strict hygienic practices even without addition of antibiotics during processing. However, if the use of antibiotics becomes inevitable, conventional antibiotics like- Penicillin and Streptomycin may still be sufficient. As for this study is concerned, only Penicillin and Streptomycin have been used which maintained the bacterial load at 260 cfu/ml of frozen semen which was very well below the permissible level (500cfu/ml) set by the Bureau of Indian Standards. However, in the event of development of antimicrobial resistance, the best results can be obtained by studying the antibiogram of bacterial isolates from semen and changing the antibiotics and their dose for incorporation during processing of frozen semen depending on the results of such study. Although in the present study the antibiotics- Gentamicin, Tylosin, Lincomycin, and Spectinomycin were found to be more sensitive and suggesting that they might be added to the semen extender for controlling bacterial load, they inherit the risk of making different bacteria resistant to these antibiotics in due course. This leaves the choice towards adopting better hygienic practices in preference to changing the antibiotics in the extender.

Summary

VI. SUMMARY

The present study was carried out with the objective to estimate the total bacterial load of semen which could contaminate the semen during various stages of handling, processing and storage.

A total of 108 semen samples (six ejaculates from each bull) from Holstein Friesian bulls stationed at Nandini Sperm Station (a unit of Karnataka Milk Federation), Kakolu, Bangalore were used in our study. The semen was collected at weekly intervals using artificial vagina and were extended in routinely used Tris egg yolk fructose glycerol extender (TEYFG).

6.1 Bacterial load of semen

The bacterial load of the semen samples was estimated by pour plate technique and 34.5% of the samples were sterile (no bacterial growth noticed). The mean values of all the bulls evaluated for the bacterial load of neat, extended and frozen semen (cfu/ml) were 3200 ±7.40, 230± 0.18 and 260± 0.82, respectively and a significant difference was found to exist between the bacterial load of neat and extended, neat and frozen semen but, not between extended and frozen semen samples.

6.2 Relationship of Methylene blue reduction time with bacterial load of neat semen

Methylene blue reduction time and the bacterial load of the neat semen was negatively and non-significantly correlated ($P<0.05$).

6.3 Antibiogram

The *in vitro* sensitivity pattern of the 36 ejaculates revealed that the bacterial colonies could be gaining resistance to routinely used Penicillin and Streptomycin (50%) and on the other hand was sensitive to Lincomycin (86.10%), Spectinomycin (81.94%), Tylosin (79.61%) and Gentamicin (76.38%).

The findings of the present study has highlighted that the bacterial contamination of semen can be minimised by adopting strict hygienic measures during collection, handling and processing of semen, while antibiotics could help in controlling the bacterial load in frozen semen but had the limitations of developing antibiotic resistant strains in the due course. The in vitro antibiogram conducted in the present study has exposed the limitations of Penicillin and Streptomycin, which have to be dispensed with, and the need for newer alternatives (antibiotics) in semen preservation.

Bibliography

VII. BIBLIOGRAPHY

ABRO, S. H., WAGAM, R., TUNIO, M. T., KAMBOH, A. A. and MUNIR, M., 2009. Biochemical activities of bacterial species isolated from the frozen semen of cattle. *J. Agri. and Social Sci***.,** **5**(4): 109-113

AHMED, K. and FOOTE, R. H., 1985. Post thaw survival and fertility of frozen bull spermatozoa treated with antibiotics and detergent. *J. Dairy Sci*., **69**: 535-541

AHMED, D., BORGOHAIN, B. N., DEKA, B. C. and DUTTA, G., 1989. Effect of Streptopenicillin and gentamicin on bacterial load and quality of frozen bull semen.*Indian Vet. J*., **66**: 886

AHMED, K. and FOOTE, R. H., 1986. Post- thaw survival and fertility of frozen bull spermatozoa treated with antibiotics and detergent. *J. Dairy Sci*., **69**: 535-541

AHMED, K. and GREESH MOHAN., 2001.Effect of antibiotics on bacterial load and quality of semen of Murrah bulls during preservation. *Indian J. Anim. Reprod*., **22**: 78- 80

AHMED, K. and GREESH MOHAN., 2002.Effect of antibiotics on the bacterial load and quality of semen of Murrah buffalo bulls at different stages of freezing.*Indian J. Anim. Sci*.**72**(2): 138-139

AHMED, K., GREESH MOHAN. and TRIPATHI, R. P., 2001. Effect of different antibiotics on semen quality during post- thaw incubation at 37°C.*Indian J. Anim. Reprod*., **22**: 81

AITKEN, R. J., 1995. Free radicals, lipid peroxidation and sperm function. *J. Reprod. Fertil.,***7**: 659-668

AJIT KUMAR, G., RAJU, P. V. and PRABHAKARAN NAIR, K., 1994. Type of bacteria and its load in fresh semen of crossbred bulls.*Indian J Anim. Reprod*., **15**(1):48-49

AKHTER, S., ANSARI, M. S., ANDRABI, S. M. H., ULLAH, N. and QAYYUM, M., 2008. Effect of antibiotic in extender on bacterial and spermatozoal quality of cooled buffalo (Bubalusbubalis) bull semen. *Reprod. Domest. Anim*., **43**: 272-278

ALA-UD-DIN, AHMAD, K. M., AKHTAR, N., QURESHI, Z. I. and AHMAD, N., 1990. Conception rates in buffaloes and cows. *Pak. Vet. J*., **10**: 36-38

ALEEM, M., CHAUDHRY, R. A., NAEEM-ULLAH RHAN, ATTA-UR-REHMAN RIZVI, and AHMAD, R., 1990.Occurrence of pathogenic bacteria in buffalo semen.*Buffalo Journal* **6**: 93-98

ALI, H., ALA-UD-DIN, SAMAD, H. A., ALI, S. and SABRI, M. A., 1994. Comparative effects of combiotic, ampicillin and gentamicin sulphate on motility percentage liveability and absolute index of liveability in the buffalo bull semen. *Pakistan Vet. J*., **14:** 223-227

ALMQUIST, J. O., GLANTZ, P. J. and SHAFFER, H. E., 1949.The effect of a combination of penicillin and streptomycin upon the livability bacterial content of bovine semen.*J. Dairy Sci*., **32**:183–90

ALTHOUSE, G. C. and LU, K. G., 2005.Bacteriospermia in extended porcine semen.*Theriogenology.*,**63**: 573- 584

ALTHOUSE, G. C., KUSTER, C. E., CLARK, S. G. and WEISIGER, R. M., 2000.Field investigations of bacterial contaminants and their effects on extended porcine semen. *Theriogenology.*, **53**: 1167- 1176

ALTHOUSE, G.C., 2008. Sanitary Procedures for the Production of Extended Semen.*Reprod. Dom. Anim*., **43** (2); 374–378

ALTHOUSE, G.C., PIERDON, M.S. and. LU, K.G., 2008. Thermo temporal dynamics of contaminant bacteria and antimicrobials in extended porcine semen. *Theriogenology.*, **70**: 1317–1323

ANDRABI, S. M. H., AHMAD, N., ABBAS, A. and ANZAR, M., 2001. Effect of two different antibiotic combinations on fertility of frozen buffalo and Sahiwal bull semen. *Pakistan Vet. J*., **21**: 166- 169

ANON., 2010.The Terrestrial Animal Health Code. Vol.1. The World Animal Health Organization (OIE).

AURICH, C. and SPERGSER, J., 2007. Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa. *Theriogenology.*, **67**(5): 912-918

AUROUX, M. R., JACQUES, L., MATHIEU, D. and AUER, J., 1991. Is the sperm bacterial ratio a determining factor in impairment of sperm motility: an in vitro study in man with Escherichia coli. *Int. J. Androl*., **14**: 264-270

BARTLETT, D. E., 1981. Bull semen: "semen microorganisms" in disease control in semen and embryos. *FAO Animal Health and Production.*, **23**:29-48

BATISH, V. K., SRINIVASAN, R. A., SARMA, P. A. and AGGARWAL, P. K., 1982.Studies on microbiological contamination of bull semen.*Annual report of National Dairy Research Institute*, Karnal., pp 46

BAUER, A. W., KIRBY, W.M. M., SHERRIS, S. C. and TURK, M., 1966.Antibiotic susceptibility testing by a standardized single disc diffusion method. *American J. Clin. Pathol.*, **45**:493

BECK, G. H. and SALISBURY, G. W., 1943.Rapid methods for estimating the quality of bull semen. *J. of Dairy Sci*., **26**: 483- 494

BHOSREKAR, M. R., 1990. Semen production and artificial insemination. BAIF, DRF *Pune, India*, *pp* 107

BIELANSKI, A., 2007. Disinfection procedures for controlling microorganisms in the semen and embryos of humans and farm animals. *Theriogenology.,* **68**: 1-22

BIELANSKI, A., BERGERON, H., LAU, P. C. and DEVENISH, J., 2003. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology.,* **46**: 46- 52

BIELANSKI, A., NADIN DAVIS, S., SAPP, T. and WALLACE, C., 2000. Viral contamination of embryos cryopreserved in liquid nitrogen. *Cryobiology*., **40**:110-116

BINDRA, D. S., 1991. Some studies on the micro flora in prepucial washing and semen of buffalo bulls. *M.V.Sc. thesis*, Punjab Agricultural University, Ludhiana, 141 004

BINDRA, D. S., PANGAWKAR, G. R., DHINGRA, P. N. and MATHAROO, J. S., 1994. Bacterial flora and their antibiotic sensitivity pattern in buffalo bull semen. *Indian Vet, J*., **71**: 460- 463

BISHOP, M. W. H., CAMPBELL, R. C., HANCOCK, J. L. and WALTON, A., 1954. Semen characteristics and fertility in bull. *J. Agric. Sci*., **44**: 227- 248 (Animal breeding Abstracts., 22: No.946)

BONADONNA, T. and SUCCI, G., 1980. Artificial insemination in the world. *Proceedings of the IX international Congress of Animal Reproduction and Artificial Insemination*, (16-20), Spain

BOUSSEAU, S., BRILLARD, J. P., MARQUANT, L., GUIENNE, B., GUERIN, B., CAMUS, A. and LECHAT, M., 1998. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology.,* **50**:699- 706

CERTIFIED SEMEN SERVICES (CSS®), 1993. CSS minimum requirement for health of bulls producing semen for AI, Missouri

CHUPIN, D., 1992. Resultatsd'uneenquetesurl'etat de l'insemination artificielle dans les pays en developpement. *Elev. Insemin*., **252**: 1–26

CHUPIN, D. and THIBIER, M., 1995. Survey of the present status of the use of artificial insemination in developed Countries. *World Anim. Rev*., **82**: 58–68

CLEMENT, F., VIDAMENT, M. and GUERIN, B., 1995.Microbial contamination of stallion semen.*Biol. Reprod.Mono*.,**1**: 779- 786.

COBO, F. and CONCHA, A., 2007.Environmental microbial contamination in a stem cell bank. *Lett. Appl. Microbiol*., **44**: 379- 86.

CRUICKSHANK, R., DUGUID, J. P., MARMAN, B. P. and SWAIN, R. H. A., 1975.*Medical Microbiology*, Churchill Livingstone, London

DANEK, J., WISNIEWSKI, E., KRUMRYCH, W. and DABROWSKA, J., 1996.Changes in the qualities of stallion semen after incubation with Streptococcus zooepidemicus. *Bulletin of the Veterinary Research Institute in Pulawy.*,**40**:111-116

de JARNETTE, J. M., MARSHALL, C.E., LENZ, D. W., MONKE, D.R., AYARS, W. H. and SATTLER, C. G., 2004. Sustaining the fertility of artificially inseminated dairy cattle: The role of the artificial insemination industry. *J. Dairy Sci.*, 87: E93-E104

DIEMER, T., WEIDNER, W., MICHELMANN, H. W., SCIEFER, H. G., ROVAN, E. and MAYER, F., 1996. Influence of *Escherichia coli* on motility parameters of human spermatozoa in vitro. *Int. J. Androl.*, **19**: 271-277

DIN, A., AHMAD, K. M., AKHTAR, N., QURESHI, Z. I. and AHMAD, N., 1990. Conception rate in buffaloes and cows. *Pakistan Vet. J*., **10**: 36- 38

EL-MULLA, K. F., KOHN, F. M. and DANDAL, M., 1996.*In vitro* effect of Escherichia coli on human sperm acrosome reaction. *Arch. Androl*., **37**: 73-78

ERB, R. E., EHLERS, M. H., MIKOTA, L. and SCHWARZ, E., 1950. The relation of simple semen quality tests to fertilising capacity of bull semen. *Wash. Agri. Expt. Sta. Tech. Bull*., ***2***. (Anim. Breed. Abstr.**58**: 587)

ERICSSON, S. A., GARNER, L.A., JOHNSON, D. REDELMAN and AHMED, K., 1990. Flow cytometric evaluation of cryopreserved bovine spermatozoa processed using a new antibiotic combination. *Theriogenology*., **33**: 1211- 1220

FACHE, M., GUERIN, B., PAREZ, M. and THIBIER, M., 1985. Etude quantitative de la florebacterienne non pathogenedans le spermeet la semence de taureau. *Elev. Insemin*., **207**: 13-18

FLASTCHER, J. and HOLZMANN, A., 1984. Genital diseases in bulls: Importance for artificial insemination control measures. In *XI Conference of the International des epizooties Regional Commission for Europe,* Vienna. (*Vet. Bull*., **56**: 3363)

FOOTE, R. H. and SALISBURY, G.W., 1948. The effect of sulphonamides upon the livability of spermatozoa and upon the control of bacteria in diluted bull semen. *J. Dairy Sci*., **31**:769–78

GANGADHAR, K. S., RAMAMOHAN RAO, A., KRISHNASWAMY, S. and UMAMAHESWARA RAO, S. M., 1986b. Bacterial load in the frozen semen of buffalo bulls. *Indian Vet. J*., **63**: 320-324

GANGADHAR, K. S., RAMAMOHAN RAO, A., KRISHNASWAMY, S. and UMAMAHESWARA RAO, S. M., 1986a. Bacterial and fungal types and their load in the frozen semen of buffalo bulls. *Indian Vet. J*., **63**(1): 48-53

GERARD, O., VISSER, I. J. R. and JANSEN, H. B., 1995. Influence of two extension procedures on the ubiquitous microflora and the biological quality of frozen bovine semen. *Reprod. Dom. Anim*., **30**: 289- 294

GIVENS, M.D. and MARLEY, M.S., 2008. Pathogens that cause infertility of bulls or transmission via semen. *Theriogenology*., **70**: 504–507

GRIVEAU, J. F., DOMOUNT, E., RENARD, P., CHALLENGANI, J. P. and LELANNOU, D., 1995. Reactive oxygen species lipid peroxidation and enzymatic defense system in human spermatozoa. *J. Reprod. Fertil*., **103**:17-26

GUNSALUS, I. C., CAMPBELL, J. J. R., BECK, G. H. and SALISBURY, G. W., 1944. The bacteriology of bull semen. II. The effect of bacteria upon rapid tests for semen quality. *J. Dairy Sci*., **27**: 357- 364

GUPTA, R. S. and MAURYA, S. N., 1993. Microbial flora of bovine semen and their antibiotic sensitivity pattern.*Indian J. Anim. Reprod*., **14**: 107- 108

HASAN, S., ANDRABI, S. M. H., MUNEER, R., ANZAR, M. and AHMAD, N., 2001. Effects of a new antibiotic combination on post-thaw motion characteristics and membrane integrity of buffalo and Sahiwal bull spermatozoa and on the bacteriological quality of their semen. *Pakistan Vet. J.*, **21**: 6-12

HUBALEK, Z., 2003. Protectants used in the cryopreservation of microorganisms. *Cryobiology*., **46**: 205-229

HUSSAIN, S. S., AHMAD, N., ALA-UD-DIN. and CHAUDHRY, N. A., 1990. Effect of different antibiotics on motility and liveability of spermatozoa and viable bacterial count in buffalo semen. *Pakistan Vet. J.*, **10**: 171-174

ISI., 1976. Indian Standards Institution. Bulletin number 8102-1976. Clause 3.3.3. Manakbhavan, 9. Bhadurshaw Zafarmarg, New Delhi

JASKO, D. J., BEDFORD, S. J., COOK, N. L., MUMFORD, E. L., SQUIRES, E. L. and PICKET, B. W., 1993. Effect of antibiotics on motion characteristics of cooled stallion spermatozoa. *Theriogenology.*, **40**(5): 885-93

JESUS, L. Y., MARIA, A., MARCO, A., JOSE, A. M. and PILAR, S., 2010. Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15^0C. *Anim. Reprod. Sci*., **122**: 142- 149

KAUR, M., TRIPATHI, K. K., BANSAL, M. R., JAIN, P. K. and GUPTA, K. G., 1986. Bacteriology of cervix in cases of infertility: effect on human sperm. *Am. J. Reprod. Immunol. Microbiol.,* **12**: 21-24

KHER, H. N. and DHOLAKIA, P. M., 1984. Bacteriological studies of bovine semen. *Indian J. Anim. Reprod*., **5**(1): 78- 84

KIM, I. H. and SON, D. S., 1998. Bacteria in semen used for IVF affect embryo viability but can be removed by stripping cumulus cell vortexing. *Theriogenology.*, **50**:293-299

KOMMISRUD, E., GRAFFER, T. and STEINE, T., 1996. Comparison of two processing systems for bull semen with regard to post thaw motility and non return rates. *Theriogenology.*, **45**: 1515- 1521

KRAUSE VON, D., WEITZE, K. F. and WABERSKI, D., 1989. Untersuchungenzurspermienvertraglichkeit von lincospectinimrahmen des in der bundesrepublikDeutshlandublichenrinderspermakonservierungsverfahrensanterverwendungeinesTriseiodotter- verdunners (Klinischekurzmitteilung).Dtsch. Tierarztl.Wochenschr.,**96**: 508- 510

KUPFERSCHMIED, H.U., BAUER, G., FLUKIGER, A. and GAILLARD, C., 1991a. Bovine insemination results after the addition of two combinations of antibiotics to the semen extender. *Reprod. Dom. Anim*., **21**: 297- 300

KUPFERSCHMIED, H. U., BAUER, G., FLUKIGER, A. and GAILLARD, C., 1991b. A new combination of antibiotics for bull semen diluents. *KB Mittelilungen*, **29**: 28-29

LINDEBERG, H., KARJALAINEN, H., KOSKINEN, E. and KATILA, T., 1999. Quality of Stallion Semen obtained by a new semen collection phantom (Equidame) versus a Missouri artificial vagina. *Theriogenology.*, **51**: 1157-1173

LORTON, S. P., SULLIVAN, J. J., BEAN, B., KAPROTH, M., KELLGREN, H. and MARSHALL, C., 1988a. A new antibiotic combination for frozen bovine semen. 2. Evaluation of seminal quality. *Theriogenology.*, **29**: 593- 607

LORTON, S. P., SULLIVAN, J. J., BEAN, B., KAPROTH, M., KELLGREN, H. and MARSHALL, C., 1988b. A new antibiotic combination for frozen bovine semen. 2. Evaluation of seminal quality. *Theriogenology*., **29**: 609- 613

MADSEN, M. and CHRISTENSEN, P., 1995. Bacterial flora of semen collected from Danish warm blood stallions by artificial vagina. *Acta Veterinaria Scandinavica,* **36**:1-7

MARINOV, P., 1988. Microbiological status of bull semen in relation to its preservation under Bulgarian conditions. *Veterinaria Sbirka.*, **86**: 46-47. (Vet. Bull. 60:2316)

MAXWELL, W. M. C. and SALAMON, S., 1993. Liquid storage of ram semen- a review. *Reprod. Fertil. Dev*., **5**:151-156

MAXWELL, W. M. C. and SALAMON, S., 2000. Storage of ram semen. *Anim. Reprod. Sci.*, **62**: 77-111

MOHANTY, P. K., MOHANTY, B. N., RAY, S. K. H., MOHANTY, D. N. and MOHANTY, L. D., 1988. Bacterial load of frozen bovine semen. *Indian Vet. J*., **65**:516- 518

MORETTI, E., CAPITANI, S., FIGURA, N., PAMMOLLI, A., GRAZIA FEDERICO, M., GIANNERINI, V. and COLLODEL, G., 2009. The presence of bacteria species in semen and sperm quality. *J. Assist. Reprod. Genet*., **26**: 47-56

MORRELL, J. M., 2006. Update on semen technologies for animal breeding. *Reprod. Domest. Anim*., **41**: 63-67

MORRIS, J., 2003. Cryopreservation and contamination. *ART and Science*., **2**: 1-3

MORRIS, J., 2005. The origin, ultrastructure, and microbiology of the sediment accumulating in liquid nitrogen storage vessels.*Cryobiology*.,**50**: 231- 238

MORRIS, J., 1999. Contamination issues. In: safe cryopreservation of gametes and embryos, McLaughlin, E., Pacey, A., Elliot, T. *Lady brook publishing*, *pp* 26

NAIDU, K.S., RAMAMOHAN RAO, A. and PANDURANGA RAO, V., 1982. Microbial flora of semen of Jersey and Murrah bulls. *Indian Vet. J*., **59**(2): 91-95

NAIK, H. S., RANA, S. K., BANDYOPADHYAY, P. K. and ROY CHOUDHURY., 1990. Studies on microbial quality of frozen semen and accessories.*Indian J. Dairy Sci*., **43**:76- 78

National project for cattle and buffalo breeding (NPCBB) report., 2006. Ministry of Agriculture Department of Animal Husbandry, Dairying and Fisheries, Government of India

NDDB Standards., 2004. Minimum standards for production of bovine frozen semen. Productivity Enhancement Group., National Dairy Development Board, Anand- 388 001

NICKOLAI, D. J., LAMMEL, C. J., BYFORD, B. A., MORRIS, J. H., KAPLAN, E. B., and HADLEY, W. K.,1985. Effects of storage temperature and pH on the stability of eleven beta-lactam antibiotics in MIC trays. *J. Clin. Micrbiol*., **21**:366- 70

OCHSENDROF, F. R. and FUCHS, J., 1993. Oxidative imbalances in male fertility.*In*: Oxidative stress in dermatology. Fuchs, Packer, L. (ed.) Marcel Dekker, New York, Basel, Hong Kong, pp. 489- 531

OSHSENDROF, F.R., 1998. Infection and reactive oxygen species. *Andrologia.*, **30**(1):81-86

OSTASZKO, F. J., 1976. In: variable fertility results in AI centres and associated factors. *Proc. 8th Int., Congress on Animal Reprod. and AI*., **5**: 1235- 1237

OTTER, A., 2008. Bacterial isolates from the semen of rams with suspected infertility. *Vet. Rec.,***162**: 623- 624

PANDA, K. B., MOHANTY, B. N., RAY, S. K. H. and MOHANTY, D. N., 1990. Effect of antibiotics on chilled bovine semen. *Indian J. Anim. Reprod*., **11**: 5-6

PAREZ, M., 1984. The most important genital diseases of cattle (control, treatment and the hygiene of semen collection). *In proc. 11th Conference of the OIE Regional Commission for Europe*, Vienna, 25-28 September. OIE, Paris, 175- 203

PICKETT, B. W. and VOSS, J. L., 1999.Physiology and philosophy of breeding horses. *J. Equine Vet. Sci*., **19**: 363-373

POMEROY, K. O., HARRIS, S., CONAGHAN, J., PAPADAKIS, M., CENTOLA, G. and BASURAY, R., 2010. Storage of cryopreserved reproductive tissues : evidence that cross-contamination of infectious agents is a negligible risk. *Fertil. Steril.,* **94**: 1181- 1188

PRABHAKAR, T. G., PREMKUMAR, D. B., MANOHAR, P, W., VENKATASUBBA RAO, C. and VENKATESAN, R. A., 1993. Bacterial analysis in the frozen semen of buffalo bulls. *Indian J. Anim. Sci*., **63**: 644- 645

RAHMAN, A., DUTTA, J. C. and RAJKONWAR, C. K., 1988. A study on preservation of buffalo semen in two extenders. *Buff. J*., **1**:57- 60

RAHMAN, A., DUTTA, J. C., BORO, B. R. and RAJKONWAR, C. K., 1983. Studies on bacteriological flora of bull semen and their antibiotic spectra.*Indian Journal of Comparative Microbiology, Immunological and Infectious Diseases.,* **4**(2):110-112

RAKESH SHARMA, K., PANGAWKAR, G. R. and MATHAROO, J. S., 1994. Efficacy of certain antibiotics on the bacterial load of buffalo semen during cryopreservation. *Indian J. Anim.* Sci., **64**: 468- 470

RAMASWAMY, V., SARAVANABAVA, K., ANDREW, M. J., VENUGOPALAN, A. T. and VENKATESAN, R. A., 1990.Microbial flora of buffalo frozen semen and their antibiogram. *Indian J. Anim. Sci*., **61**: 843- 845

RAMASWAMY, V., JOHN, K. J., JOSEPH, A. J. M., PARIMAL, R. and VENUGOPALAN, A. T., 1994. Prevalence of microbes in frozen cattle semen and their antibiotic spectra.*Indian J. Anim. Reprod*., **15**(1): 50-52

RAMASWAMY, V., LATHA, N. and GNANASUBRAMANIAN, T., 1997. Multiple drug resistant bacteria in bovine frozen semen.*Indian J*. *Animal Health.*, **36**(2): 91-96

RAMASWAMY, V., LATHA, N., GNANASUBRAMANIAN, T. and MANICKAM, R., 2002. Aerobic bacteria in buffalo semen and their antibiogram. *Indian J. Anim. Reprod*., **23**: 117-119

RATHNAMMA, D., RAO, M. S., RAMANATHA, K. R. and RAGHAVAN, R., 1997. Assessment of bacterial load in semen of Holstein Friesian bulls. *Current research Univ*. *of Agri*. *Sci*., Bangalore, **11**: 205- 207

ROBERTS, S.J., 1971. In: Veterinary obstetrics and genital diseases. *Edn 2^nd.^*,Scientific book agency, 22. Raja Woodman street, Calcutta., *pp* 708-721

RONALD, B. S. M. and PRABHAKAR, T. G., 2001b.Bacterial analysis of semen and their antibiogram. *Indian*. *J.Anim. Sci.,* **71**(9): 829-831

RONALD, B. S. M. and PRABHAKAR, T.G., 2001a.Effect of *Pseudomonas aeroginosa* and *Proteus mirabilis* on spermatozoa. *Indian J. Anim. Sci.*,**72**: 140.

RONCEANU, L., STOENESCU, A., PERUANA, T. and COMAN, T. 1973.(fide Singh. N *et al.* 1990)

SALISBURY, G. W., VAN DENMARK, W. C. and LODGE, J. R., 1978. *Physiology of Reproduction and Artificial Insemination of Cattle*. Edn 2nd. W. H. freeman and co. San Francisco. pp 286- 328

SALLE, A. J., 1974. Fundamental Principles of Bacteriology. Edn 7th., Cambridge.

SANDEEP, J., KATOCH, R. C., DEEPTI, C. and ARVIND M., 2000a. Evaluation of bacterial load in fresh ejaculates of bovine and buffalo bull semen in Himachal Pradesh. *Indian J. Anim. Sci.*, **70**(5): 465-467

SANDEEP, J., KATOCH, R. C., DEEPTI, C. and ARVIND, M., 2000b. The drug resistant bacteria associated with frozen semen technology of cow and buffalo bulls. *Indian J. Anim. Sci*., **70**: 488-489

SANSONE, G., NASTRI, M. J. F. and FABBROCINI, A., 2000. Storage of buffalo (*Bubalusbubalis*) semen. *Anim. Reprod. Sci*., **62**: 55-76

SCHULZ, M., SÁNCHEZ, R., SOTO, L., RISOPATRÓN, J. and VILLEGA, J., 2010. Effect of Escherichia coli and its soluble factors on mitochondrial membrane potential, phosphatidyl serine translocation, viability, and motility of human spermatozoa.*American Society for Reproductive Medicine Fertility and Sterility*., **94**(2): 619-623

SEMAKOV, V.G., 1963. In semen production artificial insemination, evaluation of semen, chapter VII, Edt. Bhosrekar, M. R., 1990 BIAF, DRF, Pune, India, pp 105- 126

SERGIN, N. P., 1940. The respiration of sperm. *Trd. Lab. Iskusst*. Osemen. Zivotn (mosk)., **1**: 156- 179. *Animal Breeding Abstract*.,**13**:6

SHIN, S. J., LEIN, D. H., PATTEN, V. H. and RUHNKE, H. L., 1988. A new antibiotic combination for frozen bovine semen. I. Control of mycoplasmas, ureaplasmas, *Campylobacter fetus subsp. Venerealis* and *Haemophilus somnus*. *Theriogenology.*, 29: 577- 591

SHUKLA, M. K., 2005. Correlation of microbial load of cryopreserved semen with quality of neat and cryopreserved Murrah buffalo bull semen. *Buff. Bull.,***24**(4): 84- 87

SHUKLA, M. K., 2008. Minimum standard protocols for frozen semen production. In Refresher training on Diagnostic and Therapeutic skill of Bovine reproductive problems. Department of Animal Reproduction, Gynaecology and Obstetrics, SDAU, SK Nagar

SINGH, N., SINHA, A. K., BALRAJ SINGH, SINGH, L. C., TIWARY, B. K. and SINGH, M. P., 1990. Efficacy of specific antibiotics on microbial load in chilled and frozen semen of crossbred bulls.*Indian Vet. J.,* **67**(6):531-536

SORENSON, E., 1942. The Dehydrogenase power of sperm cells as a measure for fertility of sperm. *Skand Vetidsker*., **32**:358. (Animal Breeding Abstract., 12: 198)

STOYANOV, T., 1987.Effect of antibiotics on micro flora of bull semen and its fertility. *Veterinary Medicine Nouki.,* **24**(3): 57-66 (*fide Veterinary bulletin* **57**:6029*)*

TANYILDIZI, S. and BOZKURT, T., 2003.The effect of Lincomycin-spectinomycin and sulfamethoxazole- trimethoprim on hyaluronidase activities and sperm characteristics of rams. *J. Vet. Med. Sci*., **5**: 775-780

TEDESCHI, R. and DE PAOLI, P., 2011. Collection and preservation of frozen microorganisms. *Methods Mol. Biol*., **675**: 313-326

THIBIER, M. A., 2002. Contrasted year for the world activity of the animal embryo transfer industry. *IETS Newsletter.*, **20**: 13-18

THIBIER, M. and GUERIN, B., 2000. Hygienic aspects of storage and use of semen for artificial insemination. *Anim. Reprod. Sci*., **62**:233-251

THIBIER, M., 1990a.New biotechnologies in cattle reproduction. *In*: Poomvises, P., Ingkaninum, P. *Proc. 7th Congress of the Fed. Asian Vet. Assoc*., 512–524, Bangkok, Thailand, Pattaya, Thailand

THIBIER, M., 1990b. Health supervision of AI bulls in France and requirements for international movement of semen within the EEC and for imports to the EEC. *Proc. 13th Techn. Conf. Artif. A.I. Reprod., Milwaukee, USA, NAAB.,* 24–29

UPSENSKLI, A., 1967. The determination of fertilizing ability of deep frozen bull semen from dehydrogenase activity. *Noloch. Myas. Skotov*., Mosk., **12**: 30-31 (Russ) (Anim. Breed. Abstr., 36: 14-52)

VANDEMARK, N.L., SALISBURY, G.W. and BRATTON, R.W., 1949. Oxygen damage to bull spermatozoa and its prevention by catalase. *J. Dairy Sci.,* **32**: 353–360

VARNER, D. D., SCALAN, C. M., THOMPSON, J. A., BRUMBAUGH, G.W., BLANCHARD, T. L. and CARLTON. T. M., 1998. Bacteriology of preserved stallion semen and antibiotics in semen extender. *Theriogenology.*, **50**: 559-573

WAYDA, E., 1991. Antibiotic resistance of bacterial strains isolated from bull semen. *Medycyna Veterenaryn.*, **47**(5):214- 15 (fide Veterinary Bulletin., **61**: 7978)

WIERZBOWSKI, S., 1981. Bull semen opportunistic pathogen and ubiquitary microflora pp 21-28. *In:* disease control in semen and embryos. *FAO Animal Production and Health paper*. 23. Food and Agriculture Organisation of the United Nations, Rome.

WIERZBOWSKI, S., NOWAKOWSKI, W., WAYDA, E. and KUZNIAK, S., 1984. Antibiotic level and bacterial contamination of frozen bulls semen (streptomycin, penicillin). *Medycyny Weterynaryjna,(Poland),* **40**(5): 284-287

Abstract

VIII. ABSTRACT

Contamination of the semen with bacteria could happen at various stages of processing and handling. In view of this and need for effective monitoring of semen processing it is felt important to evaluate the bacterial load. This approach enables to check the contamination points and to recommend appropriate measures to overcome the problems associated with such contamination. With the very same purpose, the present study was undertaken to estimate the bacterial load of semen at various stages of semen processing, their antibiogram profiles and suitable recommendations. According to Office Internationale Epizooticus (OIE) the standard for acceptable colony forming units is 500 CFU per ml of frozen semen. The production of frozen semen of a larger dimension needs a quality control service to maintain supply of good quality frozen semen and to attain a good quality of frozen semen microbial analysis should be carried out at each and every step of processing. The present study was undertaken at the semen collection centre – Nandini Sperm Station (a unit of Karnataka Milk Federation), Kakolu, Bangalore. Six adult healthy bulls were selected and six ejaculates from each bull at weekly intervals and subjected for the estimation of total bacterial load in the neat, extended and frozen semen to evaluate the level of contamination occurring during handling, processing and storage of semen and the bacterial load of the semen was found to be within the prescribed guidelines of BIS suggestive of the hygienic measures followed during semen collection and preservation. The antibiogram of the mixed cultures in the frozen semen samples were carried out to evaluate the efficacy of the conventionally used antibiotics and to suggest better antibiotic or combination of antibiotics as approved by the OIE and the study revealed that the conventional antibiotics used in the semen station can be sufficient to reduce the bacterial load in the semen along with strict hygienic measures during collection and processing of semen.