# EFFECTS OF ANTIBIOTICS ON MOTILITY, SPERM MORPHOLOGY, MEMBRANE INTEGRITY, FERTILITY AND BACTERIOLOGICAL QUALITY OF BUFFALO SPERMATOZOA

A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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## CERTIFICATE

This thesis by Sayed Murtaza Hassan Andrabi is accepted in its present form by the Department of Animal Sciences as satisfying the thesis requirements for the degree of Doctor of Philosophy in Animal Sciences (Reproductive Physiology).

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In the Name of

# ALLAH

The Most Beneficent,
The Most Merciful

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## THESIS ABSTRACT

The main objectives of this study were to determine the (1) bacterial species incriminated in buffalo (*Bubalus bubalis*) bull semen, (2) efficacy of a new antibiotic combination, i.e. GTLS (gentamycin, tylosin and linco-spectin) in extender on bacterial counts and appearance of bacterial genera in buffalo semen at different stages of cryopreservation, and *in vitro* drug sensitivity against the bacteria isolated from fresh semen, (3) effect of GTLS in extender on post-thaw motilities, motion characteristics, plasma membrane integrity, general and acrosomal morphology of buffalo bull spermatozoa, and (4) suitability of GTLS in extender for improvement in fertility of deep-frozen buffalo bull spermatozoa to obtain better pregnancy rate through artificial insemination. In experiment 1, a total of eleven bacterial species were identified from ejaculates of four adult buffalo bulls. The bacterial isolates identified with their frequency of occurrence (%) were; *Staphylococcus aureus* (16%), *Staphylococcus intermedius* (11%), *Staphylococcus coagulase* (8%), *Staphylococcus epidermis* (10%), *Escherichia coli* sp. (16%), *Pseudomonas aeruginosa* (13%), *Pseudomonas putida* (2%), *Pseudomonas testosteroni* (4%), *Micrococcus* sp. (11%), *Proteus mirabilis* (6%) and *Bacillus* sp. (4%). In experiment 2, total aerobic bacterial counts (cfu/ml; median) in post-thaw samples were lower ($P<0.05$) in GTLS (0.00) than in SP (streptomycin and penicillin; 0.88) or NC (negative control; 2.78). Fewer bacterial genera were identified in semen samples treated with GTLS. They were staphylococcus, micrococcus and *E. coli*. In addition to these three genera, pseudomonas,

proteus and bacillus were observed in SP treated samples. Majority of the bacterial isolates from fresh semen samples showed more sensitivity towards gentamycin, tylosin, lincomycin and spectinomycin as compared with streptomycin and penicillin. Susceptibility to lincomycin and spectinomycin was shown by 100% isolates of pseudomonas. In experiment 3, mean motilities (visual, computer-assisted linear and circular), velocities (straight-line, average path and curvilinear) and amplitude of lateral head displacement in post-thaw semen samples did not differ ($P>0.05$) due to antibiotics. Similarly, sperm plasma membrane integrity did not vary ($P>0.05$). Morphologically abnormal spermatozoa (%) were lower ($P<0.05$) in GTLS ($17.7 \pm 2.8$) and SP ($16.9 \pm 3.1$) than in NC ($28.2 \pm 3.9$). Sperm cells possessing normal acrosome (%) were higher ($P<0.05$) in GTLS ($81.2 \pm 3.1$) and SP ($79.9 \pm 3.8$) than in NC ($72.1 \pm 3.1$). In experiment 4, the fertility rates for SP-based vs. GTLS-containing frozen semen of buffalo bull were 42.8 and 55.2%, respectively. The results for GTLS were significantly higher than SP. The fertility rates also differed significantly in the first and second batch of inseminations performed with SP or GTLS-based cryopreserved semen of buffalo bull. In conclusion, bacterial and seminal quality measured by standard laboratory tests and field fertility trial indicates that GTLS combination of antibiotics is more suitable in semen extender for cryopreservation of buffalo bull spermatozoa.

## GENERAL INTRODUCTION

The Nili-Ravi buffalo, classified as river type belonging to the Murrah group, is recognized as the highest milk producing breeds of buffalo (Cockrill, 1974). Being the principal breed in Pakistan she plays an important role in the livestock industry and rural economy of the country (Economic Survey, 2005-2006).

The production potential of livestock can be increased by genetic improvement utilizing one of the modern ways of breed improvement, the artificial insemination (AI) *see also* Figure 1.

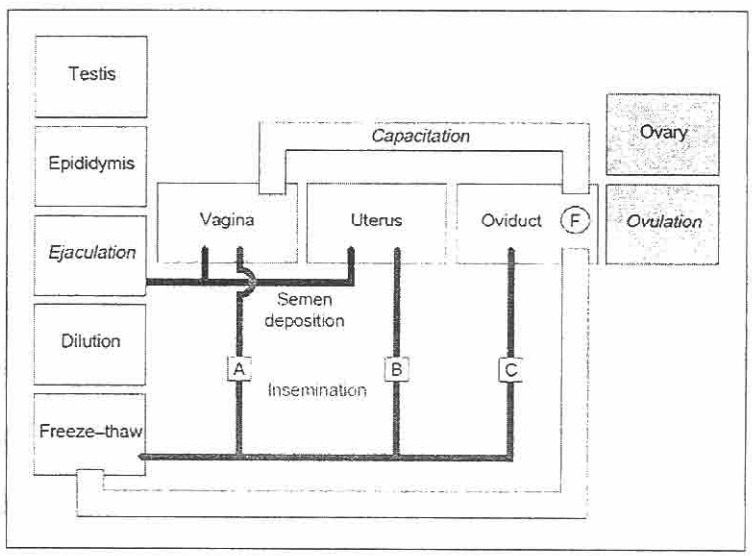


**Figure 1.** Schematic diagram illustrating the steps involved in reproduction of farm animals. Frozen-thawed spermatozoa may be inseminated into the vagina (**A**) into the uterus trans-cervically (**B**) or laparoscopically into the oviduct (**C**) (*adopted from* Curry, 2000).

Application of AI has been reported at limited scale in buffalo because of poor conception rates with frozen-thawed spermatozoa (Ahmad, 1997). Therefore, quality of frozen semen is one of the most influential factors to establish the conception rate in the field (Saacke, 1984). There are many factors known to affect the viability or fertility of cryopreserved bull spermatozoa (Curry, 2000). Factors include bacteria in semen and their control via addition of antibiotics in freezing diluents (Thibier and Guerin, 2000; Morrell, 2006).

**Bacteriospermia and its Control**

Despite the fact that even after following the standard epidemiological rule to maintain pathogen-free bull semen for AI (*illustrated in* Figure 2) the microbes may exist in the semen of the bull (Thibier and Guerin, 2000). Bacteria in bovine semen may come from testes or epididymus, the accessory glands, the vas deferens, the urethra, or the prepuce or the penis (Carballo, 1981; Marcus et al., 1994a,b; Thibier and Guerin, 2000). Extenders having ingredients of animal origin (egg yolk or milk) can also be source of bacteria, consequently resulting in contamination of semen (Bousseau et al. 1998; Marco-Jimenez, 2004; de Ruigh et al., 2006). Even under careful conditions semen may get contaminated at the time of collection or subsequent handling/packaging -'dip and wipe' procedure- (Russell et al., 1997; Thibier and Guerin, 2000; Holt, 2000; Kapoor, 2003) or even during storage in deep-frozen state (Brown et al., 1974; Bielanski et al., 2003; Mazzilli et al., 2006).

Table 1 and 2 enlists bacteria isolated from fresh and frozen buffalo bull semen, respectively.

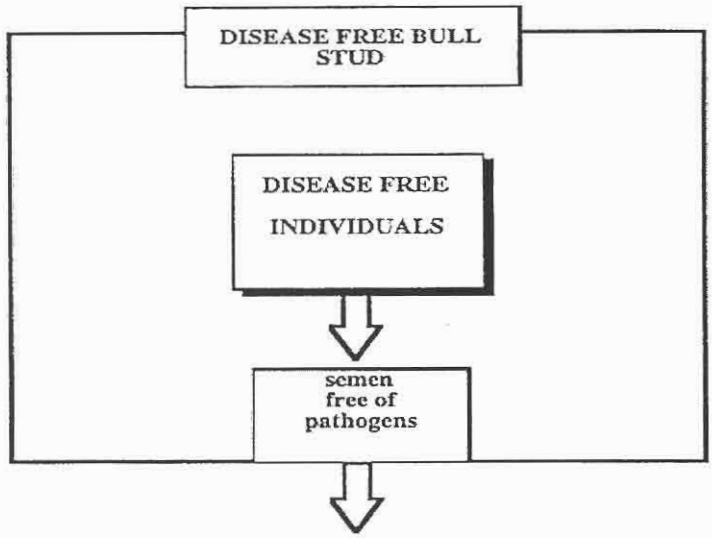


**Figure 2.** The epidemiological rule to maintain pathogen-free bull semen for AI (*adopted from* Thibier, 1990).

It is documented that the semen containing substantial number of bacteria usually have leukocytes and polymorphonuclear granulocytes (de Geyter et al., 1994; Shukla et al., 2005), and this causes production of reactive oxygen species (ROS) (Ochsendrof, 1998). Increased ROS levels impair the sperm functions and ultimately their fertilizing capacity (Hammerstedt et al., 1990; Aitken, 1995; Griveau et al., 1995; Maxwell and Stojanov, 1996; Dalvit et al., 1998; Chatterjee and Gagnon, 2001; Raina et al., 2002). Also the presence of micro-organisms, especially the bacteria in the ejaculates can affect fertilization directly (Morrell, 2006), by adhering to spermatozoa (Bolton et al.,

**Table 1.** Bacteria isolated from fresh buffalo bull semen.

| Naidu et al., 1982 | Pal et al., 1989 | Aleem et al., 1990 | Pal et al., 1993 | Bindra et al., 1994 | Sharma et al., 1996 | Amin et al., 1999 | Jasial et al., 2000 | Ahmed et al., 2001 |
|---|---|---|---|---|---|---|---|---|
| *Bacillus sp.* | *Mycoplasma bovigenitalium* | *Escherichia coli* | *Mycoplasma bovigenitalium* | *Escherichia coli* | *Acholeplasma genus* | *Chlamydia psittaci* | *Staphylococci* | *Corynebacterium b* |
| *Micrococcus sp.* | *Mycoplasma bovis* | *Pseudomonas aeruginosa* | *Acholeplasma laidlawii* | *Pseudomonas genus* | *Ureaplasma genus* | | *Micrococci* | *Corynebacterium pyogenes* |
| *Pseudomonas sp.* | *Mycoplasma bovis* | *Pseudomonas putida* | | *Corynebacterium genus* | | | *Corynebacteria* | *Corynebacterium r* |
| *Alcaligenes sp.* | *Mycoplasma arginini* | *Pseudomonas fluorescens* | | *Staphylococcus aureus* | | | *Entero-bacteriaceae* | *Staphylococcus au* |
| *Staphylococcus aureus* | *Acholeplasma laidlawii* | *Pseudomonas testosteroni* | | | | | *Anthracoids* | *Streptococcus pyogenes* |
| *Escherichia coli* | | *Staphylococcus aureus* | | | | | | *Pseudomonas aeruginosa* |
| *Corynebacterium pseodiptheriticum* | | *Proteus mirabilis* | | | | | | *Pseudomonas pyocyanae* |
| *Corynebacterium equi* | | *Streptococcus pyogenes* | | | | | | *Enterobacter cloac* |
| *Proteus sp* | | *Enterobacter sp.* | | | | | | *Listeria monocytog* |
| *Staphylococcus sp.* | | *Shigella sp.* | | | | | | *Proteus vulgaris* |
| | | *Micrococcus sp.* | | | | | | *Alcaligenes faecal* |
| | | *Diphtheroids* | | | | | | *Citrobacter freundi* |
| | | | | | | | | *Bacillus sp.* |

**Table 2.** Bacteria isolated from frozen buffalo bull semen.

| Rao et al., 1980 | Ramachandra et al., 1981 | Roslanowski et al., 1985 | Gangadhar et al., 1986 | Ramaswamy et al., 1997 | Ramaswamy et al., 2002 | Kapoor, 2003 |
|---|---|---|---|---|---|---|
| *Acinetobacter calceaceticus* | *Acinetobacter calceaceticus* | *Campylobacter sputorum* | *Enterobacter liquefasciens* | *Bacillus sp.* | *Bacillus sp.* | *Bacillus sp.* |
| | | | *Pseudomonas aeruginosa* | *Corynebacterium sp.* | *Corynebacterium sp.* | *Corynebacterium renale* |
| | | | | *Staphylococcus sp* | *Staphylococcus sp.* | *Streptococcus pyogenes* |
| | | | | | *Pseudomonas aeruginosa* | *Staphylococcus aureus* |
| | | | | | *Escherichia coli* | *Staphylococcus epidermidis* |
| | | | | | *Aeromonas sp.* | *Pseudomonas aeruginosa* |
| | | | | | *Streptococcus sp.* | |

1986; Wolff et al., 1993; Diemer et al., 1996), impairing their motility (Panangala et al., 1981; 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). Microbes transmitted through semen can also result in abortions and infections of female genital tract (Wentink et al., 2000; Bielanski et al., 2000).

Effect of sperm or ovum interaction with specific micro-organism has been studied for some bacteria. Gram-negative micro-organisms are reported to attach themselves to spermatozoal membranes (Bolton et al., 1986). Wolff et al. (1993) elucidated that *Escherichia coli* adhered to sperm surface via mannose binding sites. Diemer et al. (1996) reported that binding of the *Escherichia coli* resulted in ultrastructural damage to sperm plasma membrane. Huwe et al. (1998) reported that *Escherichia coli* lowered sperm motility *in vitro*. *Escherichia coli* is also associated with uterine infections and altered fertility in cows (Griffin et al., 1974a,b; Del Vecchio et al., 1994; Dhaliwal et al., 2001; Foldi et al., 2006). *Staphylococcus aureus* is reported to alter sperm function *in vitro* by interfering with the metabolic processes (Borcyckzo, 1982). *Pseudomonas aeruginosa* can cause serious genital diseases in AI bulls (Borcyckzo, 1982) and interfere with the sperm cell viability and probably lower the conception rate too (Eaglesome and Garcia, 1995; Corona et al., 2006). Also if *Pseudomonas aeruginosa* is transmitted to the female's genital tract at the time of breeding, it can result in infectious endometritis and associated sub-fertility (Varner et al., 1998). Aleem et al.

(1990) reported that the 48 hours old cultures from buffalo semen of *Pseudomonas aeruginosa*, when injected to mice through intra-peritoneal route, killed them within 24 hours. *Proteus mirabilis* is often implied in infertility cases of equines (Clement et al., 1993). Eaglesome and Garcia (1990) studied the effect of *Mycoplasma bovis* on the ability of bull spermatozoa to interact with zona free hamster oocytes (ZFHO) in an *in vitro* assay. The addition of *Mycoplasma bovis* to both unextended and extended semen at a mycoplasma to sperm cell ratio of 10:1 significantly reduced sperm penetration rates and the mean number of sperm per penetrated egg. Similarly, the ability of spermatozoa to form pronuclei and the activation of penetrated oocytes were adversely affected by *Mycoplasma bovis*. Moreover, when *Mycoplasma bovis* was added to the oocytes, there was a marked reduction in the sperm penetration rates and fertilization processes suggesting that the organism affects certain oocyte function(s). Therefore, the results indicate that the presence of *Mycoplasma bovis* in semen or in the female reproductive tract may affect fertilization. Lee et al. (1997) identified *Pseudomonas aeruginosa*, *Acinetobacter calceaceticus* and *Flavobacterium sp.* in frozen- thawed cattle (*Bos taurus*) bull semen that influenced *in vitro* fertilization (IVF) badly in terms of cleavage and development to morulae or blastocysts. Recently, Sylla et al. (2005) evaluated whether *Mycoplasma mycoides* ssp. *mycoides* LC contaminated bovine ejaculates could impair *in vitro* fertilizing ability of bull spermatozoa and *in vitro* embryo development. Progressive motility decreased significantly upon spermatozoa incubation with *Mycoplasma*. Also, *Mycoplasma* contamination reduced calcium ionophore

treatment efficacy (P<0.05). Ultrastructurally, *Mycoplasma* microorganisms appeared as moderately electron-dense sphere-shaped particles, adhering to cell membranes. Sperm mid-piece sections showed numeric aberrations of the central singlets. Further morphological abnormalities included partial or total absence of dinein arms and radial fibers, with lack of the bridge and the central ring, whereas these abnormalities were not observed in uninfected ones. The IVF trials showed that two–four cell blocks were higher ($P < 0.05$) in the infected group. Ultrastructure of Day-7 contaminated embryos showed *Mycoplasma* particles adhering and infiltrating the outer layer of the zona pellucida. These investigations suggest that *M. mycoides* ssp. *mycoides* LC contaminating the bovine ejaculate induced adverse effects on *in vitro* spermatozoa-fertilizing ability and embryonic development. Besides bacteria, the protozoan, *Trichomonas foetus*, transmittable through mating and/or semen is characterized by infertility and abortion in bovine (Wentink et al., 2000).

Use of antibiotics in semen extenders to control several micro-organisms from bulls or from contamination during semen processing, provided major contribution for the development of AI (Zaugg and Almquist., 1973; Ahmad et al., 1987; Gerard et al., 1995; de Jarnette et al., 2004). Streptomycin and penicillin (SP) were among the first to be researched intensively for preservation of bovine semen. They were found to be relatively harmless to sperm cells and, particularly in combination, inhibited a broad spectrum of micro-organisms (Salisbury and VanDemark, 1961; Maule, 1962). Almquist

(1948) first reported that 500 to 1000 IU of penicillin per ml of extended semen increased the fertility of low-fertility cattle bulls. Foote and Bratton (1950) obtained higher non-return (NR) rates when 1000 $\mu$g of streptomycin per ml, or 1000 IU of penicillin per ml, or both, were added to semen extender. These findings were substantiated by other reports (Almquist, 1951; Willett and Larson, 1952).

The addition of SP in the semen extender which has been historically used in AI industry was questioned as these antibiotics were not useful against corynebacterium, pseudomonas, vibrios, brucellae, mycobacterium, mycoplasmas, ureaplasmas and haemophilus (Roberts, 1971; Shin et al., 1988). Moreover, some of the organisms which were previously sensitive to SP became resistant to these antibiotics (Alford, 1953; Parusov, 1974; Ahmad and Foote, 1985). Regarding control of bacteriospermia in buffalo bull semen, Gangadhar et al. (1986) found that SP was not a very effective combination. Aleem et al. (1990) examined the micro-organisms present in buffalo bull semen, and their *in vitro* sensitivity to commonly available antibiotics. They found that SP was ineffective in controlling the bacteria isolated from buffalo bull semen. Hussain et al. (1990) and Ali et al. (1994) studied the effect of different antibiotics in extender on quality of buffalo bull semen. They concluded that the combination of SP was relatively inefficient for preservation of buffalo bull semen. Similarly, Amin et al. (1999) found that the standard combination (SP) was ineffective to control *Chlamydia psittaci* in processed buffalo bull semen.

The failure to control bacteriospermia in bovine necessitated the screening of wide array of antibiotics for their effects on semen quality and fertility (Ahmad, 1987 - PhD Thesis Cornell University, Ithaca; *also published as* Ahmad and Foote, 1985; Ahmad and Foote, 1986; Ahmad et al., 1987). Ahmad (1987) measured percentage motility and percentage of intact acrosome in frozen-thawed cattle bull semen, and fertility following AI, to evaluate the spermicidal effect of the antibiotics (amikacin, dicloxacillin, cephapirin, ceforanide, gentamycin, minocin, tiamulin, or a combination of amikacin and cephapirin) added to extenders [(whole milk (WM), egg yolk-Tris (EYT) or egg yolk-Tris-sodium and triethanolamine lauryl sulphate (STLS)]. He found that Amikacin (500 $\mu$g/ml) did not depress motility or 1st service NR rate (70.4 in the treated vs. 70.5% in the control). Cephapirin or ceforanide (200 $\mu$g/ml) in extenders or cephapirin (1000 $\mu$g/ml) in raw semen were non-toxic. The NR rate for semen containing cephapirin (500 $\mu$g/ml) in 2 trials was 75.5 and 72.1% compared with 72.1 and 72.7% in the controls. The motility of semen in extenders containing dicloxacillin or tiamulin at 500 $\mu$g/ml was lower ($P<0.05$) than in the control semen without antibiotics (52 vs. 50%). Tiamulin also depressed the percentage of intact acrosome. Minocin was highly toxic when used in EYT and STLS extenders but not in WM. Reagent grade gentamycin (1000 $\mu$g/ml) and tiamulin (100 $\mu$g/ml) were also effective in all extenders, as was minocin (100 $\mu$g/ml) in WM. In an another experiment, Ahmad (1987) added gentamycin, clindamycin, amikacin, minocin, linco-spectin, tylosin or tiamulin to raw cattle bull semen, the non-glycerol portion of egg yolk-citrate (EYC), EYT or WM. Gentamycin in EYT (250 $\mu$g/ml), tylosin in EYC (500 $\mu$g/ml), and

amikacin in either EYT (250 $\mu$g/ml) or WM (500 $\mu$g/ml) depressed ($P<0.05$) semen motility after thawing. Clindamycin in all extenders (50 $\mu$g/ml) was spermicidal ($P<0.05$) while tiamulin was innocuous. Minocin (500 $\mu$g/ml) was non-toxic in WM, but spermicidal in EYT and EYC extenders. Linco-spectin (300/600 $\mu$g/ml) was more ($P<0.05$) spermicidal in WM than in EYC or EYT extenders. From the first experiment it was concluded, that cephapirin and amikacin are the antibiotics of choice for use in extenders for bovine semen, while from the second experiment it was concluded that that all antibiotics (gentamycin, clindamycin, amikacin, minocin, linco-spectin, tylosin), with the exception of clindamycin, can be used to control contaminating organisms in bovine semen. It is also clear that the kind and quality of antibiotic, the time and temperature of exposure of micro-organisms to the antibiotics, and the composition of extender should be considered in formulating a satisfactory protocol for controlling bacteriospermia in bovine semen (Truscott and Abreo, 1977; *Salisbury et al.*, 1978).

Stoyanov (1987) studied the effect of eight antibiotics (gentamycin, amoxicillin, kanamycin, polymyxin, oxytetracycline, chloramphenicol, streptomycin and penicillin) in media on thawed-pelleted cattle bull semen. It was found that gentamycin, amoxicillin, kanamycin, and polymyxin preserved the semen cells and enhanced their survival rate at the same level as that of the control medium with no antibiotic. Streptomycin and penicillin in combination lowered 2.5 times spermatozoal motility. Worst was the survival rate of spermatozoa when the medium was supplemented with

oxytetracycline and chloramphenicol. It was also found that best sanitation qualities with regard to the micro-flora in semen had gentamycin, which, at that did not produce any negative effect on the rate of survival. The conception rate of cows inseminated with semen treated with gentamycin at thawing was shown to be 15.3% higher, and when treated with SP, it was 3.0% higher at first insemination than that of the control group of animals inseminated with semen with no antibiotic supplement.

Later, some of the selected antibiotics were systematically studied, for their dose, method of addition and interaction with the extender and their effects on microbes (Shin et al., 1986; Shin et al., 1988). This led to the development of a new combination (gentamycin, tylosin and linco-spectin; GTLS) for cryopreservation of cattle bull semen (Doak, 1986). It has been demonstrated that the combination of GTLS (added to semen extender at the rate of gentamycin 500 µg/ml, tylosin 100 µg/ml and linco-spectin 300/600 µg/ml) is more effective for controlling micro-organisms including mycoplasmas, ureaplasmas, *Campylobacter fetus*, *Haemophilus somnus*, and pseudomonas in bovine semen than other antibiotics or combination of antibiotics added to extenders (Shin et al., 1986; Shin et al., 1988; Guerin and Thibier, 1993). Also systemic studies of this antibiotic combination (GTLS) have revealed that it is not detrimental to post-thaw quality or fertility of cattle bull semen (Lorton et al., 1988a,b; Sullivan et al., 1988; Krause et al., 1989; Ericsson et al., 1990; Kupferschmied et al., 1991a,b; Gerard et al., 1995; Bousseau et al., 1998). To my knowledge no published systematic reports on affect of addition of GTLS

to freezing diluents on viability or fertility and bacterial quality of buffalo bull semen seems to be available. It is relevant to mention that conventionally penicillin 1000 IU/ml and streptomycin sulphate 1000 $\mu$g/ml alone or in combination is commonly added to freezing diluents of buffalo bull semen (Sansone et al., 2000).

In summary, micro-organisms are usually present in the ejaculates of bovine. The aim of obtaining sterile semen is almost unachievable. Thus, in the use of AI, it is important to efficiently control the population of micro-organisms in the semen. However, to attain this, it has been necessary to clearly identify the agents that pose the main risks and not only those of intrinsic pathogenicity, but also those likely to contaminate and lead to a high concentration of micro-organisms in the preserved semen. Effect of the relatively new antibiotic combination (GTLS) in extender on semen quality or fertility has been widely assessed in cattle (*Lorton et al., 1988a,b; Kupferschmied et al., 1991a,b*; Guerin and Thibier, 1993; Kommisrud et al., 1996; Bousseau et al., 1998), while no information is available in case of water buffalo. Thus testing of GTLS systematically could make a meaningful contribution in the improvement of quality and fertility of cryopreserved buffalo bull semen.

**Semen Quality**

High quality semen is a key for success to both high production and reproduction in bovine (Rasul, 2000). However, inadequate accuracy in

semen quality assays in the laboratory (Graham and Crabo, 1978), and inability to measure fertility with reliability (Saacke et al., 1980) are the two major problems related to the association of semen quality and fertility.

**Sperm function tests**

The assessment of spermatozoal quality is one of the major concerns in semen research and AI (Rasul, 2000). Presently, neither a single post-thaw laboratory assay will enable the determination of *a priori* of the potential fertility level that the analysed semen will reach, particularly after AI (Rodriguez-Martinez, 2003). However, the combination of several assays may better predict fertility (Linford et al., 1976; Graham et al., 1990; Foote, 2003). The common viability related assays used for the assessment of semen quality include motility, velocity, penetration of cervical mucus, metabolic activity, cell contents, ability to agglutinate (head to head) in the presence of blood serum, ability to pass through sephadex-glass wool filter and structural integrity of cell membrane and acrosome (Saacke, 1984; Correa et al., 1997; Tanghe et al., 2002). However, the combination of these assays has provided the equivocal results with fertility. For instance, correlations between sperm motility and fertility ranged from as low as 0.15 to as high as 0.83 (Kjaestad et al. 1993; Bailey et al. 1994; Stalhammar et al. 1994; Januskauskas et al. 2003). Even analyses of standardized semen samples from bulls using CASA (computer-assisted semen analyzer) instrument have shown variable correlations between certain patterns of post-thaw motility, such as linearity,

and field fertility ($r^2$ = 0.45–0.63; Zhang et al. 1998; Januskauskas et al. 2001). Combined statistical analyses of various motility patterns yielded stronger correlations ($r^2$ = 0.68–0.98; Farrell et al. 1998) and predictive values were presented when the outcome of motility assessments were combined with other parameters of sperm function ($r^2$ = 0.83; Januskauskas et al. 2001) in bulls. Correlations between sperm morphology and fertility have also been found to vary widely ($r^2$ = 0.06 to 0.86; Graham et al. 1980), but were most often statistically non-significant when the semen of AI-quality grade was assessed (Rodriguez-Martinez et al. 1997; Saacke 1999).

Despite difficulties in correlating laboratory data with fertility, laboratory evaluation of semen quality remains important. Although laboratory semen analyses may not predict actual fertilizing potential of a semen sample, the analysis may predict samples of low fertility (Amann, 1989; Graham, 1996; Colenbrander et al., 2003; Amann, 2005). Fertility of semen can also be established with AI through NR or return rates to oestrus (Foote, 2003; Rodriguez-Martinez, 2003). However, the proper estimation of fertility rates requires large sets of NR rates, e.g., large numbers of inseminations (in hundreds or thousands) per semen batch or bull (Rodriguez-Martinez, 2003).

Assays used in the present studies on buffalo bull semen will be discussed in this part of thesis.

Sperm motility

Motility of spermatozoa has been shown to be required for colonization of the

isthmus of oviduct (Hunter and Wilmut, 1984). Sperm motility is an important criterion for determining the quality of sperm population, both before AI, and in the laboratory to evaluate the effect of experimental procedures (Figure 3). Sperm motility is routinely estimated visually, by the use of closed circuit television (Graham et al., 1970). Unfortunately, visual determination is highly a subjective assessment method, depends on observer's accuracy, and semen handling techniques (Graham, et al., 1980; McNiven et al., 1992). Furthermore, it is not a reliable assay for predicting fertility in bulls (Saacke and White, 1972; Linford, et al., 1976; Brahmkshtri et al., 1999).

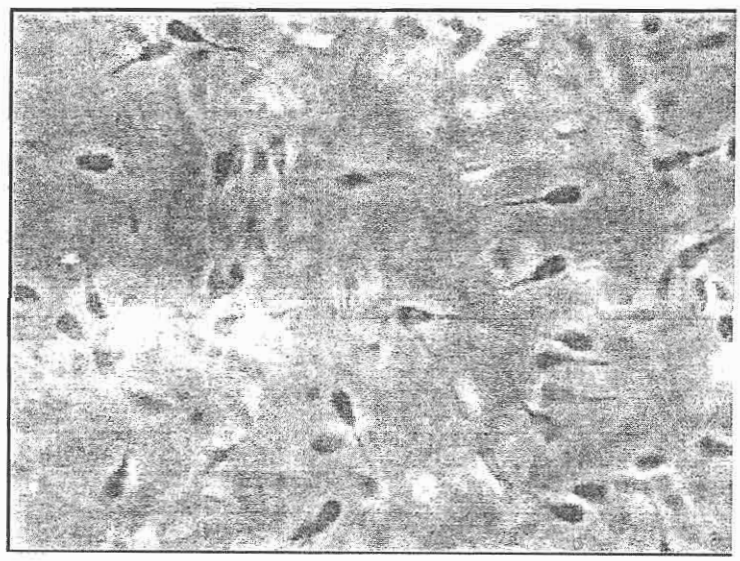

**Figure 3.** Glimpse of motility of buffalo bull semen under phase contrast microscope (x400).

For the last several decades, different approaches have been used, to develop the objective methods for the evaluation of sperm motion characteristics. The

objective assessments of sperm motility are reasonably accurate and repeatable, based either upon massive movement of spermatozoa (Rothschild, 1948; Glover, 1968) or the measurement of proportion of motile spermatozoa and individual velocities of those spermatozoa (Harvey, 1945; Rothschild, 1953; Katz and Dott, 1975).

The techniques used for the objective assessment of spermatozoa included, microcinematography (David et al., 1981), videomicrography (Katz and Overstreet, 1981; Johnson et al., 1981), time-exposure photomicrography (Rothschild, 1953; Overstreet et al., 1979; Aitken et al., 1985), multi-exposure photography (Makler, 1978), and laser-doppler spectroscopy (Cooke and Hallett, 1976; Hartmann et al., 1983). However, the use of computer-assisted sperm motility was carried out almost two decades earlier (Liu and Warme, 1977; Amann, 1979). This system was found inaccurate when the samples contain less than 25 percent motile spermatozoa (Amann and Hammerstedt, 1980). Concomitantly, O'Connor et al. (1981) concluded that with that system there is no advantage over conventional laboratory tests for the prediction of fertility of cryopreserved bull spermatozoa.

In bulls, Budworth et al. (1988) reported, by the use of CellSoft™ computer system, a good correlation ($r^2 \geq 0.68$; $P<0.05$) between the competitive fertility index and the percentage of motile spermatozoa, linear velocity and straight-line velocity. Likewise, Amann (1989) reported an $r^2$ value of 0.86 between the percentage of motile sperm and a competitive fertility index. With the

development of modified forms of computerized semen analysis significant correlation with fertility have shown to predict fertilization rates (Check et al., 1990; Fetterolf and Rogers, 1990).

Sperm motion characteristics

The details of sperm motion characteristics can provide insights into the physiology of cells, the mechanisms of their transport, and fertilization (Yanagimachi, 1978; Katz and Overstreet, 1980). With the advancement in computer technology, software has been developed that capture a series of video images in digital form, in real time, and provides comprehensive data on sperm motion. This system is highly dependent on its settings (Knuth et al., 1987) and has been used successfully in different species (Budworth et al., 1988; Ellington et al., 1993; Jasko et al., 1993; Berger et al., 1994; Rasul et al., 2000) due to its more precision.

Computer analysis of sperm velocity offers a rapid, objective and predictive assessment of sperm function. Progressive velocity and the actual movement of the spermatozoa have been shown to be closely related with sperm penetration into cervical mucus (Aitken et al., 1985; Mortimer et al., 1986), heterologus ZFHO penetration test (Aitken et al., 1982a; 1982b), and IVF (Jeulin et al., 1986). Sperm velocity and amplitude of lateral head displacement (ALH), as measured by CASA, appear to be better predictors of an individuals' fertility, than is the percentage of progressively motile sperm cells in a semen sample of bull and human (Ellington et al., 1993). The measurement of velocity has been

considered as an indirect indicator of mitochondrial function of a spermatozoon (Graham et al., 1984) and is associated with fertility (Budworth et al., 1988). Amplitude of lateral head displacement may indirectly reflects the efficiency of the flagellar beat pattern, appears to be a critical factor whenever the generation of shearing forces is involved, as in sperm penetration of cervical mucus or their ability to penetrate the cumulus oophorus and the zona pellucida (Jeulin et al., 1986). It was poorly correlated with their ability to fuse with ZFHO (Aitken et al., 1985). The schematic model of sperm velocities and ALH is presented in Figure 4.

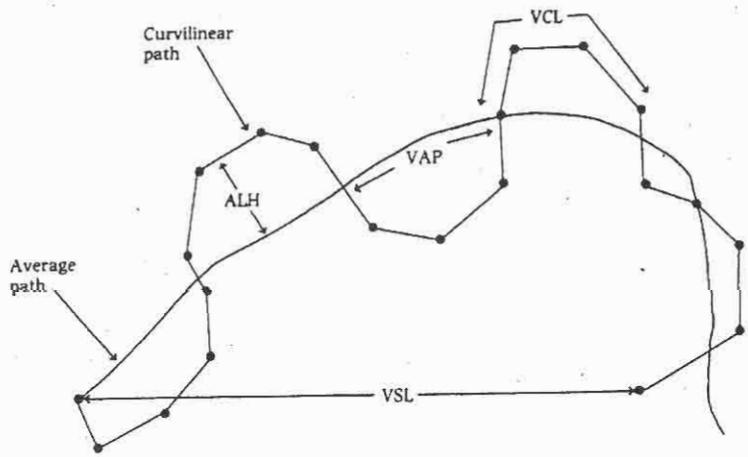


**Figure 4.** Schematic representation of the different velocities and lateral head displacement as measured by the computer-assisted sperm analysis systems. VSL = straight line (or progressive) velocity, VAP = average path velocity, and VCL = curvilinear velocity, ALH = amplitude of lateral head displacement (*adopted from* Amann, 1988; Verstegen et al., 2002).

Sperm plasma membrane integrity

The plasma membrane surrounds the entire sperm cell holding together its organelles and intracellular components and by its semi-permeable features maintains the chemical gradient of ions and other soluble components (Silva and Gadella, 2006). The sperm plasmalemma plays a pivotal role in controlling sperm fertilizing ability; not only does it directly mediate the contact interactions between the spermatozoa and the oocyte itself, but it also act as the receiver of the environmental signals that induce the positive response the spermatozoon must make in order to achieve fertilization. Due to this reason the integrity and functional activity of the plasma membrane of spermatozoa is of fundamental importance in the fertilization process. Its assessment may be a useful indicator to determine the fertilizing ability of spermatozoa.

The evaluation of plasma membrane integrity is of particular importance due to its involvement in metabolic exchanges with the surrounding medium. Furthermore, the process of capacitation, acrosome reaction and the oocyte penetration requires a biochemically active membrane (Jeyendran et al., 1984). Plasma membrane integrity determined by hypo-osmotic swelling (HOS) stress test has a good correlation to fertility ($r^2$ = 0.90; Jeyendran et al., 1984) and sperm morphology (Van Der Van et al., 1986) in human, and to sperm motility ($r^2$ = 0.73) in bull (Correa and Zavos, 1995). A significant correlation has been reported between the HOS-assay and bovine mucus penetration test ($r^2$ = 0.77), the progressive motility ($r^2$ = 0.70), and sperm morphology ($r^2$ = 0.51) in human (Gehring, 1987).

Several assays have been developed to assess the functional integrity of spermatozoa besides the standard evaluating methods including adenosine triphosphate of sperm, cervical mucus penetration, hemizona attachment assay, ZFHO penetration assay, triple staining, and sperm acrosin activity (Toda et al., 1992). The assessment of live and dead spermatozoa by supravital staining is mainly carried out with the help of eosin-nigrosin stain. Upon staining the clear head indicates an intact membrane that resists the stain and the broken or inactive membrane absorbs the stain (Swanson and Bearden, 1951). The use of HOS-assay has frequently been reported for different species (Bredderman and Foote, 1969; Jeyendran et al., 1984; Kumi-Diaka, 1993; Correa et al., 1997) including buffalo (Azam et al., 1998; Rasul et al., 2000; Ahmad et al., 2003). The HOS-assay is a simple, quick and inexpensive method to assess functional integrity of plasma membrane (Jeyendran et al., 1984; Kumi-Diaka, 1993). The HOS test assesses the functional integrity of the sperm membrane in the tail region and in-directly provides information about membrane associated cell functions (permeability, ion-exchange, $O_2$ transport, function of membrane associated enzymes; Figure 5) (Rasul, 2000).

Zavos (1990) has suggested HOS-test as a valuable assay for the assessment of plasma membrane damage during the cryopreservation cycle. Exposure to spermatozoa to a hypo-osmotic solution causes an influx of water through the plasmalemma until an osmotic equilibrium is reached, resulting in a swollen cell, mainly manifesting itself as a curled sperm tail. The plasma membrane surrounding the tail fibres appear to be more loosely attached than the

membrane surrounding the head, so that the tail region shows the swelling more clear (Jeyendran et al., 1984). Sperm tail swelling generally indicates that the transport of water across the plasma membrane has occurred normally, an indicative of normal membrane integrity and function as reflected by the ability of the spermatozoa to undergo capacitation and fusion with ZFHO (Jeyendran et al., 1984).

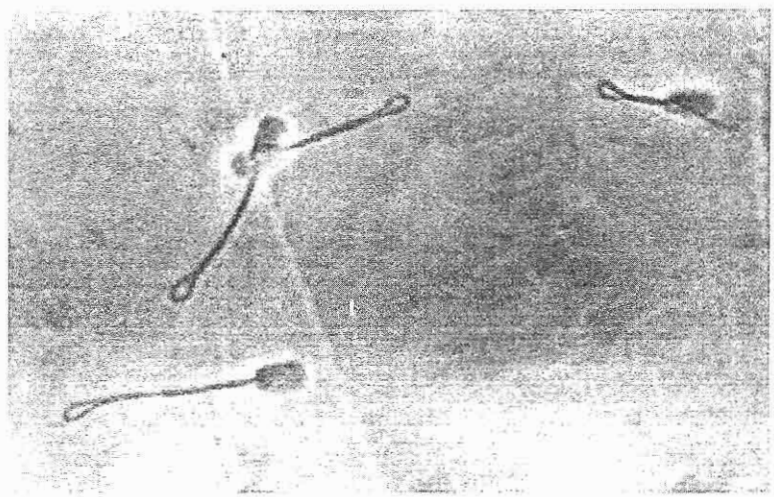

**Figure 5.** Hypo-osmotic swollen spermatozoa of buffalo bull characterized by coiling of tail (x400).

Sperm acrosome membrane integrity

The acrosome is a large Golgi/endoplasmic reticulum derived acidic secretory organelle. It is filled with hydrolytic enzymes that are organized in a kind of enzyme matrix and most enzymes are heavily glycosylated (Ramalho-Santos et al., 2002). The presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is being required at the proper time

to facilitate fertilization (Thomas et al., 1997). The change in acrosomal cap is mainly due to sperm aging or injury (Saacke and Marshall, 1968), which can be effectively determined by fixing the specimen using phase contrast microcsopy (Pontbriand et al., 1989; Anzar and Graham, 1993; Azam et al., 1998; Rasul et al., 2000; Ahmad et al., 2003). Different acrosomal abnormalities were reported in semen from certain subfertile bulls (Saacke et al., 1968). Therefore, acrosomal integrity assessment is an effective tool to monitor the integrity of bull spermatozoa.

Correlation of intact acrosome with fertility in bovine has been reviewed by Graham et al. (1980), Saacke (1984) and Coulter (1992). The highest correlation between the percentage of intact acrosome and fertility of frozen bovine spermatozoa was observed after 2 and 4 hours of post-thaw incubation (Saacke and White, 1972). The acrosomal integrity determination has relatively good relationship with fertility ($r^2$ = 0.60; P<0.01) than with abnormal sperm morphology ($r^2$ = –0.27 to –0.37; P<0.01) of cryopreserved bull semen (Saacke and White, 1972). It has been reported in a heterospermic study (Saacke et al., 1980) that the percentage of intact acrosome was highly correlated with the competitive fertility index ($r^2$ = 0.90, P<0.01). Sperm acrosomal integrity was positively correlated with fertility in bulls (Brendtson et al., 1981; Pace et al., 1981; Karabinus et al., 1990). Whereas, in a recent study, percent sperm with intact acrosome was not significantly correlated with the conception rate in bulls (Brahmshtri et al., 1999).

Several methods are available to determine the percentage of acrosome-reacted sperm in a semen sample. Most of them are complicated or require expensive reagents and equipment. Techniques such as indirect immunofluorescence assays that use a monoclonal antibody to acrosomal proteins have been developed (Byrd and Wolf, 1986; Rajamahendran et al., 1994). Alternatively, histochemical stains have been used (Bryan and Akruk, 1977; Lenz et al., 1983; Lenz et al., 1988). Lectins labeled with fluorochromes have proven useful for sperm from many species (Cross et al., 1985; Cummins et al., 1986). Chlortetracycline fluorescence patterns and electron microscopy also indicate acrosomal status (Saling et al., 1979; Lee et al., 1987; Perez et al., 1996a, b). More, recently the use of Coomassie Blue G-250 staining method has been shown to be a reliable method for the assessment of acrosomal status in a variety of species (Larson and Miller, 1999). In bovine, the acrosome is large enough to be identified by routine microscopy (Sanchez et al., 1995), by differential interference contrast microscopy (Johnson et al., 1976), or by phase-contrast microscopy (Bamba and Cran, 1988; Rasul et al, 2000; Ahmad et al., 2003) *see also* Figure 6.

Determination of sperm acrosome membrane integrity by differential interference contrast microscopy or by phase-contrast microscopy has provided a valuable criterion for evaluating sperm quality in bulls (Saacke and Marshall, 1968; Saacke, 1970). However, the use of phase-contrast microscopy due to less expensive and easier to operate over differential interference contrast microscopy for the routine study of bull sperm

cytomorphology has been recommended (Galli et al., 1989) and will be used in one of the present studies.

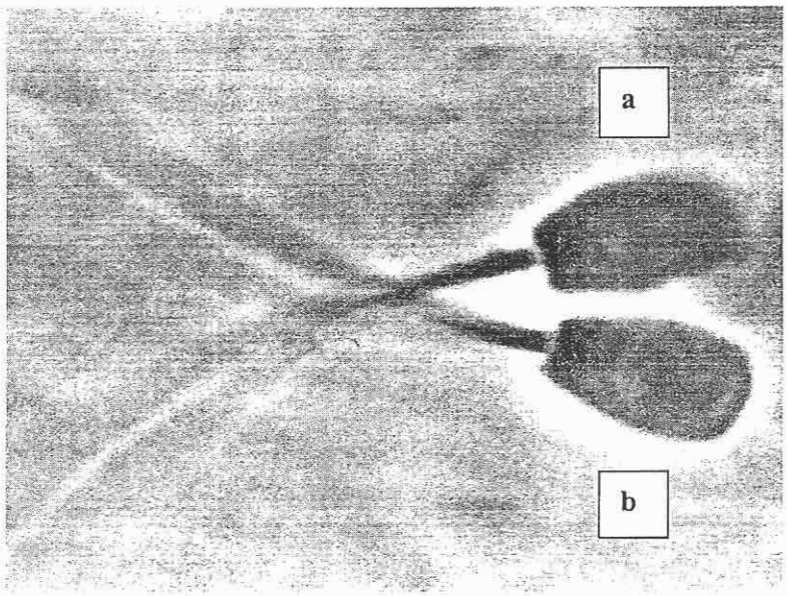


**Figure 6.** Acrosome morphology of buffalo bull spermatozoa **(a)** missing acrosome, **(b)** intact acrosome (x1000).

Sperm morphology

Evaluation of sperm morphology is one of the commonest methods to assess viability of frozen-thawed semen (Rocha et al., 2006). Different types of morphological abnormal spermatozoa have been classified either into major and minor defects (Blom, 1973) or primary and secondary defects (Saacke and White, 1972) *see also* Figure 7 and 8.

Defects which have been associated with reduced fertility in bovine with AI

are abnormal heads, coiled tails and proximal droplets (Linford et al., 1976, Wood et al., 1986; Soderquist et al., 1991). Embryo studies have produced conflicting evidence on the effect of abnormal heads on fertility. Saacke and others (1998) found fewer accessory sperm with abnormal heads than were

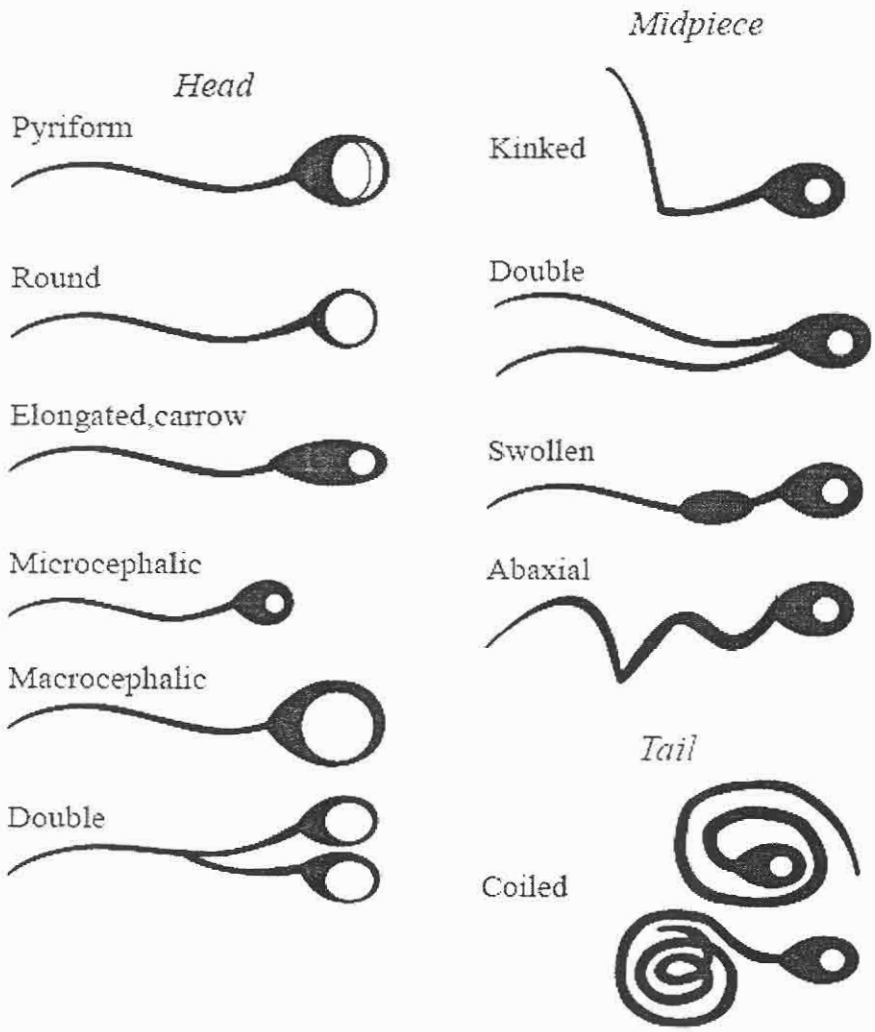


**Figure 7.** Primary sperm abnormalities of bovine semen (*adopted from* Whittier and Bailey, 2000).

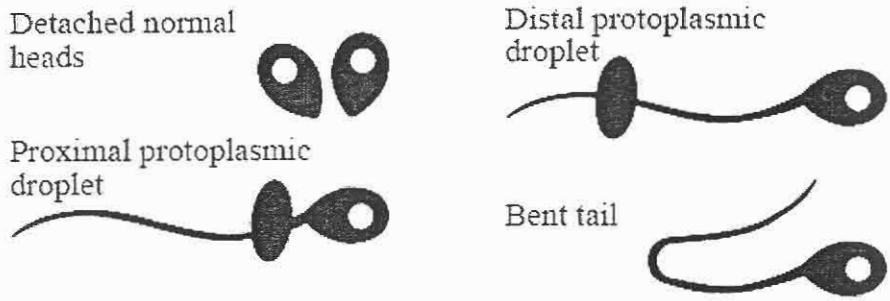


**Figure 8.** Secondary sperm abnormalities of bovine semen (*adopted from* Whittier and Bailey, 2000).

present in the inseminated population. The more severe types appeared to have been excluded, possibly as a result of some being 'dead'. Thundathil et al. (1988) , using semen from a bull with 85% of sperm with pyriform heads, observed a reduction in fertility but no increase in embryo loss, and de Jarnette et al. (1992) reported an increase in degenerate and low-quality embryos associated with semen containing high proportions of sperm with abnormal heads. The former may be the effect of grossly abnormal heads and the latter of more subtle defects. A study of 34 ejaculates from 24 bulls found fewer abnormal heads, detached heads, coiled tails and proximal cytoplasmic droplets/pseudodroplets in the frozen-thawed semen than in the raw semen (Revell, 2003). Also there was no change in the number of bent tails in frozen-thawed and raw semen, however, the number of distal cytoplasmic droplets/pseudodroplets increased in thawed semen (Revell, 2003). A significant correlation has also been established between the fertility of frozen-thawed bull semen and the frequencies of some sperm abnormalities,

particularly those related to sperm head forms and the presence of proximal cytoplasmic droplets (Soderquist et al., 1991).

Various techniques have been used to assess sperm morphology in the bull, including light microscopy of stained and unstained preparations, and transmission or scanning electron microscopy (Barth and Oko, 1989). However, use of phase-contrast microscopy appears to be the most practical method for evaluating sperm morphology (Lindford et al. 1976; Chacon et al., 2001).

Estimating fertility with artificial insemination

Although microscopic evaluation of semen quality is assessed either subjectively or objectively with the aid of hi-tech computer soft ware, the techniques cannot be an alternate for estimating fertility through AI. In technologically advanced countries, fertility in bovine AI is the return or NR to service. For this a record system was devised in the early days of AI to report all inseminations to the organization producing the semen, so the return or NR for service was available on all animals inseminated (Foote, 2003).

In the early days of AI, before antibiotics were used and when venereal diseases were prevalent, there was a major decline in the percentage of returns to service between 4 and 5 weeks after service and later returns, partly because of early embryo mortality (Foote and Bratton, 1952; Alexander et al., 1995). Antibiotics greatly reduced this loss, and bull variance was

reduced. Subsequently, the early estimates of pregnancy were highly correlated with later estimates ($r^2$ = 0.97), and by 5 months these NR estimates closely followed (within 5%) calving rates in cows (Foote, 1978).

When estimating fertility with AI it should also be considered that a female does not become pregnant after a single service tells little about the true fertility of the male (Muller, 2000); this is true because there are many factors causing a zygote not to develop into a fetus detectable by rectal palpation or ultrasonography (Amann, 2005). Some causes of embryo death are associated with the fertilizing spermatozoon (Saacke et al., 2000), but most are associated with the ovum, environment within the female reproductive tract, physiology of the female, oestrus detection or timing of AI (Amann, 2005). Consequently, failure of pregnancy does not allow a conclusion if the male, female, or both were responsible for the failure. Pregnancy, on the other hand, is evidence that the female provided a satisfactory ovum and environment (Muller, 2000), and that at least a few sperm displayed the full repertoire of the attributes (Amann and Hammerstedt, 1993) necessary to survive in the oviduct and fertilize an ovum (Amann, 2005).

Another major factor impairing our ability to accurately estimate fertility is the number of spermatozoa present in the AI dose (Rodriguez-Martinez, 2003). When bull semen has been cryopreserved, the apparent fertility of the majority of AI bulls follows a hyperbolic dose-response curve in relation to the total number of viable, normal spermatozoa. Such a curve reaches an

individual plateau of maximal fertility (usually below 100%) after showing a marked increase that also follows a different slope for each individual animal (Pace et al. 1981). There is a statistically significant relationship between the total number of viable spermatozoa inseminated (but not their percentage in the AI dose) and the fertility post-AI, following linearly the fertility level for each sire (Shannon and Vishwanath 1995; Den Daas et al. 1998). This means that the insemination of additional spermatozoa from a subfertile bull would not necessarily result in better fertility than the one already shown (Pace et al. 1981; Den Daas et al. 1998). For this reason, it is advisable to build a dose-response curve, which is used by some AI companies in developed countries, for each sire where the sperm numbers in the AI dose that is required for maximum fertility is usually between 5 and 20 million spermatozoa (Rodriguez-Martinez, 2003).

As mentioned that estimation of fertility with AI is dependent on the accuracy of oestrus detection or timing of AI and the reporting system. Furthermore, fertility rates should be corrected for several factors, such as inseminator, moment of insemination, category of female considered, season, geographical area, etc., in order to minimize the influence of external factors (Rodriguez-Martinez, 2003). When it is well managed, fertility that is based upon NR rates is considered highly repeatable and heritable ($r^2$ = 0.69, h2¼0.55, Shannon and Searle 1962). Also, large numbers of animals are needed to average out the extraneous variation associated with insemination of each female (Oltenacu et al., 1980). If only 10 females are inseminated, for

example, the variation for the fertility data will be about 25% (Amann, 1989; Amann, 2005). Therefore, if 5 of 10 females become pregnant, true fertility of that semen sample could range from 25 to 75% (Amann, 1989; Amann, 2005). In order to determine a true fertility percentage with a variation of approximately 10%, nearly 75 females per treatment must be inseminated, and at least 100 females must be inseminated to reduce the variation to 7% (Amann, 1989; Amann, 2005).

My current work is based upon thorough investigation of bacterial control and improvement of seminal quality via antibiotics in extender for cryopreservation of buffalo bull spermatozoa.

The main objectives of my study were

1. To determine the bacterial species incriminated in buffalo bull semen.

2. To determine the effect of a new antibiotic combination, i.e. GTLS (gentamycin, tylosin and linco-spectin) in extender on bacterial counts and appearance of bacterial genera in buffalo semen at different stages of cryopreservation, and *in vitro* drug sensitivity against the bacteria isolated from fresh semen.

3. To determine the effect of GTLS in extender on post-thaw motilities, motion characteristics, plasma membrane integrity, general and acrosomal morphology of buffalo bull spermatozoa.

4. To determine the suitability of GTLS in extender for improvement in fertility of deep-frozen buffalo bull spermatozoa to obtain better pregnancy rate through AI under field conditions.

# CHAPTER 1

## ISOLATION OF BACTERIAL SPECIES IN BUFFALO BULL SEMEN

## ABSTRACT

This study was carried out to determine the bacterial species incriminated in buffalo bull semen. Twenty ejaculates (n=20), five each from four healthy Nili-Ravi buffalo bulls, were obtained in an artificial vagina (42°C) over a period of five weeks. The ejaculates were examined for bacterial species by using standard microbiological techniques. A total of eleven bacterial species were identified from ejaculates of four adult buffalo bulls. The bacterial isolates identified with their frequency of occurrence (%) were; *Staphylococcus aureus* (16%), *Staphylococcus intermedius* (11%), *Staphylococcus coagulase* (8%), *Staphylococcus epidermis* (10%), *Escherichia coli* sp. (16%), *Pseudomonas aeruginosa* (13%), *Pseudomonas putida* (2%), *Pseudomonas testosteroni* (4%), *Micrococcus* sp. (11%), *Proteus mirabilis* (6%) and *Bacillus* sp. (4%). In conclusion, a number of bacterial species were isolated from buffalo bull semen and the predominant bacteria were *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Therefore, effective antibiotics are required in extender for their control during cryopreservation of buffalo bull semen.

## INTRODUCTION

Artificial insemination (AI) can be used as a powerful tool for buffalo breed improvement, thus increasing the utilization of superior sires. Application of AI has been reported at limited scale in buffalo because of poor conception rates with frozen-thawed spermatozoa (Ahmad, 1997). Therefore, for successful AI, quality of frozen semen is pivotal to establish an acceptable conception rate in the field (Saacke, 1984). There are many factors known to affect the viability or fertility of cryopreserved bull spermatozoa (Curry, 2000). Factors include bacteria in semen and their control via addition of antibiotics in freezing diluents (Thibier and Guerin, 2000; Morrell, 2006).

Bacteria in semen may come from testes or epididymis, the accessory glands, the vas deferens, the urethra, or the prepuce or the penis (Carballo, 1981; Marcus et al., 1994a,b; Thibier and Guerin, 2000). Even under careful conditions semen may get contaminated at the time of collection or subsequent handling/packaging (Kapoor, 2003; Russell et al., 1997; Thibier and Guerin, 2000; Holt, 2000) or even during storage (Brown et al., 1974; Bielanski et al., 2003; Mazzilli et al., 2006). In case semen used for AI contains micro-organisms, it can lower the conception rate or even disseminate diseases among healthy population (Eaglesome and Garcia, 1995; Thibier and Guerin, 2000).

Buffalo is the main livestock breed of Pakistan, however, the literature reveals that there is only a single published report by Aleem et al. (1990) on the microbial flora of buffalo bull semen in the country. Furthermore, the aim of obtaining sterile semen is almost unachievable. Thus, in the use of AI, it is important to efficiently control the population of micro-organisms in the semen. However, to attain this, it has been necessary to clearly identify the agents that pose the main risks and not only those of intrinsic pathogenicity, but also those likely to contaminate and lead to a high concentration of micro-organisms in the preserved semen. Therefore, a detailed study was carried out to determine the bacterial species incriminated in buffalo bull semen.

## MATERIALS AND METHODS

### Semen collection

Twenty first ejaculates, five each from four health Nili-Ravi buffalo bulls (P22, JG1, P25 and P4) maintained at Livestock Research Station, National Agricultural Research Centre, Islamabad, Pakistan were used in the study. Semen was collected in artificial vagina at 42°C at weekly intervals. The frequency of collection from each bull was two consecutive ejaculates per week.

### Microbial analysis

Samples from fresh semen were cultured for aerobic bacteria on blood agar (Gibco, Madison, WI) using standard techniques (Merchant and Pecker, 1967). In order to obtain pure culture, the colonies, which appeared after 24 hours of incubation at 37°C, were selected on the basis of their morphological characteristics and again cultured on blood agar. Isolates were typed by using Biolog® GP2 & GN2 MicroPlate kit system by swabbing the bacteria from the surface of the agar plate, and then suspending to a specific density in GN/GP Inoculating Fluid (Biolog®). After that 150 $\mu$l of bacterial suspension was pipetted into each well of the GP2/GN2 MicroPlate. The MicroPlate was incubated at 35°C for 24 hours. Finally, the MicroPlates were read visually and compared to the GN/GP Database to determine the bacterial species.

# RESULTS

## Occurrence of bacterial species

Prevalence of different bacterial species identified in each Nili-Ravi buffalo bull's semen is presented in Table 1. A total of eleven bacterial species were identified from ejaculates of four buffalo bulls. The bacterial isolates identified with their frequency of occurrence (%) were; *Staphylococcus aureus* (16%), *Staphylococcus intermedius* (11%), *Staphylococcus coagulase* (8%), *Staphylococcus epidermis* (10%), *Escherichia coli* sp. (16%), *Pseudomonas aeruginosa* (13%), *Pseudomonas putida* (2%), *Pseudomonas testosteroni* (4%), *Micrococcus* sp. (11%), *Proteus mirabilis* (6%) and *Bacillus* sp. (4%). The predominant bacteria were *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* in ejaculates of buffalo bulls.

**Table 1.** Occurrence of bacterial species in buffalo bull semen.

| ull | Isolates† | Frequency of appearance (%) of bacterial species | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (n) | *S. aureus* | *S. intermedius* | *S. coagulase* | *S. epidermis* | *E .coli sp.* | *P. aeruginosa* | *P. putida* | *P. testosteroni* | *Microco-ccus sp.* | *P. mirabilis* | *Bacillus sp.* |
| 22 | 13 | 5 | 16 | 4 | 7 | 21 | 14 | 3 | 9 | 12 | 2 | 7 |
| G1 | 8 | 21 | 17 | 9 | 13 | 14 | 12 | - | 3 | - | 9 | 2 |
| 25 | 17 | 11 | 2 | 14 | 19 | 8 | 9 | 2 | 3 | 24 | - | 8 |
| 4 | 11 | 27 | 9 | - | 2 | 21 | 17 | 5 | - | 7 | 12 | - |
| otal | 49 | 16 | 11 | 8 | 10 | 16 | 13 | 2 | 4 | 11 | 6 | 4 |

†Isolates represent the colonies, which appeared after 24 hours of incubation at 37°C.

## DISCUSSION

The bacterial species isolated in the present study from fresh Nili-Ravi buffalo bull semen are in agreement with the results of Naidu et al. (1982) and Aleem et al. (1990), who isolated same types of bacteria from Murrah and Nili-Ravi buffalo bull semen, respectively. The isolation of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas testosteroni* and *Proteus mirabilis* in the present study is significant because of their deleterious effects on viability of buffalo bull spermatozoa as has been reported by other workers (Naidu et al., 1982; Aleem et al., 1990; Ramaswamy et al., 1997; Shukla et al., 2005). Bacteriospermia appear to exert their spermicidal effect directly upon the sperm cell (Althouse and Lu, 2005).

The results of present study and that of others (Brown et al., 1974; Palli et al., 1975; Rahman et al., 1983; Saika et al., 1987; Pal et al., 1989; Aleem et al., 1990; Ronald and Prabhakar, 2001) indicate a similarity in bacterial ecology of buffalo semen with that of cattle bull (Thibier and Guerin, 2000). The frequency of occurrence of bacteria isolated in this study is in agreement with that of Raghavan et al. (1971), Paspelov et al. (1973) and Aleem et al. (1990). The predominance of *Staphylococcus aureus* (16%), *Escherichia coli* (16%) and *Pseudomonas aeruginosa* (13%) is of great significance because of their negative impact on vitality of spermatozoa (Kapoor, 2003). *Staphylococcus aureus* is reported to alter sperm function *in vitro* by interfering with the

metabolic processes (Borcyckzo, 1982). Interaction of sperm with *Escherichia coli*, a contaminant in human semen, has been one of the most extensively studied (Auroux et al., 1991; Wolff et al., 1993; Monga and Roberts, 1994; Diemer et al., 1996). Wolff et al. (1993) elucidated that *Escherichia coli* adhered to sperm surface via mannose binding sites. Diemer et al. (1996) reported that binding of the *Escherichia coli* resulted in ultrastructural damage to sperm plasma membrane. Huwe et al. (1998) reported that *Escherichia coli* lowered sperm motility *in vitro*. *Escherichia coli* is also associated with uterine infections and altered fertility in cows (Griffin et al., 1974a,b; Del Vecchio et al., 1994; Dhaliwal et al., 2001; Foldi et al., 2006). *Pseudomonas aeruginosa* can cause serious genital diseases in AI bulls (Borcyckzo, 1982) and interfere with the sperm cell viability and probably lower the conception rate too (Eaglesome and Garcia., 1995; Corona et al., 2006). Also if *Pseudomonas aeruginosa* is transmitted to the female's genital tract at the time of breeding, it can result in infectious endometritis and associated subfertility (Varner et al., 1998). Aleem et al. (1990) reported that the 48 hours old cultures from buffalo semen of *Pseudomonas aeruginosa*, when injected to mice through intra-peritoneal route, killed them within 24 hours. The isolation of *Proteus mirabilis* in the present study from buffalo bull semen is also of great significance, because it is often implied in infertility cases of equines (Clement et al., 1993).

It is also suggested that the kinds of bacteria found in semen under a given set of circumstances depends on the microbial population available to invade the genital tract (and also, therefore, on management of bulls) as well as on

the ability of bacteria to survive conditions in the preputial cavity and urethra (Brown et al., 1974).

In conclusion, a total of eleven bacterial species were isolated from buffalo bull semen and the predominant bacteria were *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. Therefore, effective antibiotics are required in extender for their control during cryopreservation of buffalo bull semen.

# CHAPTER 2

## EFFECT OF A NEW ANTIBIOTIC COMBINATION ON BACTERIAL COUNTS AND APPEARANCE OF BACTERIAL GENERA IN BUFFALO SEMEN AT DIFFERENT STAGES OF CRYOPRESERVATION, AND *IN VITRO* DRUG SENSITIVITY AGAINST THE BACTERIA ISOLATED FROM FRESH EJACULATES

## ABSTRACT

This study was carried out to determine the efficacy of a new antibiotic combination, i.e. GTLS (gentamycin, tylosin and linco-spectin) in extender for control of bacteria in buffalo bull semen. The corollary objective was to determine the effect of GTLS on the total aerobic bacterial counts (TABC), on appearance of bacteria isolated in semen samples at different stages of cryopreservation, and *in vitro* drug sensitivity against the isolated bacteria from fresh semen. Ten first ejaculates (n=10), five each from two healthy buffalo bulls, were obtained in an artificial vagina (42°C). Semen ejaculates, possessing at least 60% sperm motility, were split and diluted in Tris-citric acid (TCA) extender (at 37°C; $50\times10^6$ spermatozoa/ml), containing either GTLS (gentamycin 500 µg/ml, tylosin 100 µg/ml and linco-spectin 300/600 µg/ml) or streptomycin 1000 µg/ml and penicillin G 1000 IU/ml (SP), or negative control with no antibiotics (NC). After dilution, the semen was cooled to 4°C in 2 hours and equilibrated for 4 hours. Semen was then packaged in 0.5 ml straws and frozen in programmable cell freezer. Frozen semen was thawed at 37°C for 45 seconds. Total aerobic bacterial counts and the frequency of appearance of bacterial genera in samples were determined in neat semen, after dilution, and after freezing/thawing using standard microbiological techniques. After identification, each bacterial isolate of fresh semen was subjected to *in vitro* antibiotic sensitivity test, against penicillin G, streptomycin, gentamycin, tylosin, lincomycin and spectinomycin. Total aerobic bacterial counts (cfu/ml; median) in post-thaw samples were lower

($P<0.05$) in GTLS (0.00) than in SP (0.88) or NC (2.78). Fewer bacterial genera were identified in semen samples treated with GTLS. They were staphylococcus, micrococcus and *E. coli.* In addition to these three genera, pseudomonas, proteus and bacillus were observed in SP treated samples. Majority of the bacterial isolates from fresh semen samples showed more sensitivity towards gentamycin, tylosin, lincomycin and spectinomycin as compared with streptomycin and penicillin. Susceptibility to lincomycin and spectinomycin was shown by 100% isolates of pseudomonas. Therefore, it can be concluded that GTLS efficiently controlled the bacteria in buffalo bull semen.

## INTRODUCTION

Use of antibiotics in semen extenders to control several micro-organisms from bulls or from contamination during semen processing, provided major contribution for the development of artificial insemination (AI) (Zaugg and Almquist., 1973; Ahmad et al., 1987; Gerard et al., 1995; de Jarnette et al., 2004). The antibiotics, streptomycin and penicillin (SP) are useful additions to extenders for bull semen and have become standard components in AI industry (Foote and Bratton, 1950; Almquist, 1951; Myers and Almquist, 1951). However, due to prolonged use of these antibiotics some strains of bacteria appear to be resistant to these antibiotics (Alford, 1953; Parusov, 1974; Ahmad and Foote, 1985). Moreover it has been reported that these antibiotics have no or little effect against some specific isolates of buffalo bull semen (Aleem et al., 1990; Ali et al., 1994). The viable bacterial count did not differ in the buffalo semen diluted with ampicillin, kanamycin or SP, however, the sperm motility was better due to ampicillin (Hussain et al., 1990). This observation was substantiated by Ali et al. (1994) who reported that motility of buffalo bull spermatozoa was highest with extenders having ampicillin followed by gentamycin and SP. Now the conventional antibiotics like SP or ampicillin have been replaced by gentamycin, tylosin and linco-spectin (GTLS; Certified Semen Services (CSS®), 2002) in cattle bull semen extender, as they are believed to be superior in controlling micro-organisms, and are not detrimental to seminal quality or fertility (Hamdy and Miller, 1971; Shin et al., 1988; Lorton et al., 1988a,b; Sullivan et al., 1988; Krause et al., 1989;

Ericsson et al., 1990; Kupferschmied et al., 1991a,b; Guerin and Thibier, 1993; Gerard et al., 1995; Bousseau et al., 1998).

This study was, therefore, proposed to determine the efficacy of a new antibiotic combination, i.e. GTLS in extender for control of bacteria in buffalo bull semen. The corollary objective was to determine the effect of GTLS on the total aerobic bacterial counts (TABC), on appearance of bacteria isolated in semen samples at different stages of cryopreservation, and *in vitro* drug sensitivity against the bacteria isolated from fresh semen.

## MATERIALS AND METHODS

### Preparation of extenders

Tris-citric acid (TCA) was used as the buffer for the experimental extenders. It consisted of 1.56 gm citric acid (Fluka, Switzerland) and 3.0 gm tris–(hydroxymethyl)-aminomethane (Sigma, St. Louis, MO) in 74 ml distilled water. The pH of buffer was 7.0 and the osmotic pressure was 320 mOsmol/Kg. Egg yolk (20%; vol/vol), fructose (0.2%; wt/vol; Merck, F.R Germany) and glycerol (6%; vol/vol; Merck, F.R Germany) were added to each of the three experimental extenders.

First extender was a combination of four antibiotics (GTLS). It comprised of gentamycin available as gentamycin sulphate (Gibco, Madison, WI) 561 µg/mg, which was added at rate of 500 µg/ml, tylosin tartrate (Elanco, Indianapolis, Indiana) was added at the rate of 100 µg/ml and linco-spectin commercially available as lincomycin hydrochloride (Upjohn Co, Kalamazoo, MI) 50 mg/ml, and spectinomycin sulphate (Upjohn Co, Kalamazoo, MI) 100mg/ml, added at the rate of 300/600 µg/ml (Shin et al., 1988). Second extender (SP) contained streptomycin (Sigma, St. Louis, MO) available as streptomycin sulphate 761 IU/mg added at the rate of 1000 µg/ml and penicillin (Antibiotics, Mianwali, Pakistan) available as benzyl penicillin 500,000 IU added at the rate of 1000 IU/ml. Third extender (NC) did not contain antibiotics and served as negative control.

## Semen collection and evaluation

Ten first ejaculates, five each from two health Nili-Ravi buffalo bulls (P4 and JG1) maintained at Livestock Research Station, National Agricultural Research Centre, Islamabad, Pakistan were used in the study. Semen was collected in artificial vagina at 42°C at weekly intervals. The frequency of collection from each bull was two consecutive ejaculates per week. Each ejaculate was transferred to the laboratory within a minute and visual motility was assessed by using phase contrast microscope (400X; Olympus BX 40) attached with a closed circuit television. Sperm concentration was assessed by digital photometer (Dr. Lange LP 300 SDM, Germany). Semen samples possessing more than 60% motility were used. The semen was given a holding time of 10-15 minutes at 37°C in water bath before dilution.

## Semen processing

Each semen sample was diluted at 37°C in a single step with one of the three experimental extenders in order to contain approximately $50\times10^6$ spermatozoa/ml. After dilution, the semen was cooled to 4°C in 2 hours @ 0.275°C/minute and equilibrated for 4 hours at 4°C. Semen was then filled in 0.5 ml straws with suction pump at 4°C in the cold cabinet unit (Minitub, Germany) and frozen in programmable cell freezer (KRYO 10 series III, UK) from 4°C to -15°C at the rate of 3°C/minute and from -15°C to -80°C at the rate of 10°C/minute. Straws were then plunged into liquid nitrogen (-196°C) and stored.

After 24 hours, semen straws were thawed at 37°C for at least 45 seconds for assessment of post-thaw semen quality.

**Microbial analysis**

Total aerobic bacterial counts

Total aerobic bacterial counts (TABC) in semen samples were determined by surface plate method (Merchant and Pecker, 1967) in fresh semen, after dilution, and after freezing/thawing. Two-fold serial dilutions were prepared in normal saline solution. Each suspension (100 μl) of the serial dilution was cultured for aerobic bacteria on tryptose agar (Gibco, Madison, WI). After 24 hours of incubation at 37°C, colonies (not more than 300/supension) were counted with the help of colony counter. TABC (cfu/ml) was calculated by counting total number of colonies in 100 μl × dilution factor × 10. The data on TABC, because of heterogeneity in the counts were transformed in log and expressed as medians.

Identification of bacteria

Samples from fresh semen, after dilution, and after freezing/thawing were cultured for aerobic bacteria on blood agar (Gibco, Madison, WI) using standard techniques (Merchant and Pecker, 1967). In order to obtain pure culture, the colonies, which appeared after 24 hours of incubation at 37°C, were selected on the basis of their morphological characteristics and again cultured on blood agar. Isolates were typed by Gram-staining and standard biochemical tests.

Antibiotic sensitivity

After identification of each isolate from fresh semen samples, they were subjected to *in vitro* antibiotic sensitivity test, against penicillin G (10 IU, Oxoid, Hampshire, England), streptomycin (10 $\mu$g, Oxoid, Hampshire, England), gentamycin (10 $\mu$g, Oxoid, Hampshire, England), tylosin (10 $\mu$g, Oxoid, Hampshire, England), lincomycin (15 $\mu$g, Oxoid, Hampshire, England) and spectinomycin (10 $\mu$g, Oxoid, Hampshire, England). Results of antibiotic sensitivity were interpreted according to the zone diameter standard set up by Koneman et at. (1983).

**Statistical analysis**

Effect of antibiotics in semen extender on TABC at different stages of cryopreservation of buffalo bull was analyzed by the analysis of variance (ANOVA) (MINITAB® Release 12.22, 1998). A $P<0.05$ was considered significant. Values for TABC were transformed into log.

## RESULTS

### Total aerobic bacterial count

The data on effect of antibiotics on TABC (cfu/ml; median) in samples of fresh semen, after dilution and after freezing/thawing in buffalo bulls are presented in Table 1. The TABC varied ($P<0.05$) due to antibiotics but did not differ ($P>0.05$) between the two bulls. This count in post-thaw samples was lowest ($P<0.05$) in GTLS (0.00), intermediate in SP (0.88), and highest in NC (2.78).

### Frequency of appearance of bacterial genera

The effect of antibiotics on frequency of appearance of bacterial genera, in semen samples of fresh, after dilution, and after freezing/thawing of buffalo bulls are presented in Table 2. Fewer bacterial genera were identified in semen samples treated with GTLS. They were staphylococcus, micrococcus and *E. coli*. In addition to these three genera, pseudomonas, proteus and bacillus were observed in SP treated samples. All bacterial genera which were identified in GTLS and SP samples were present in NC samples.

### Resistance of bacteria to antibiotics

The *in vitro* sensitivity of bacteria isolated from fresh semen samples of buffalo bulls to antibiotics are presented in Table 3. A total of 26 bacterial isolates of different genera from fresh semen of buffalo bulls were tested. Susceptibility to

streptomycin was shown by 100% isolates of staphylococcus and bacillus, whereas isolates of micrococcus showed 62% susceptibility to streptomycin. Sensitivity to gentamycin was made by all the isolates (100%) of staphylococcus, proteus and bacillus. Susceptibility to tylosin (100%) was shown by isolates of micrococcus and *E. coli*. All the isolates of staphylococcus, *E. coli* and pseudomonas were susceptible (100%) to lincomycin. Susceptibility to spectinomycin was shown by 100% isolates of pseudomonas and bacillus. Overall, all the bacterial isolates were more susceptible to gentamycin, tylosin, lincomycin and spectinomycin than streptomycin and penicillin G.

**Table 1.** Effect of antibiotics in semen extender on TABC cfu/ml at different stages of cryopreservation in buffalo bulls.

| Bull | Stage | Treatment | TABC cfu/ml | | |
|---|---|---|---|---|---|
| | | | Median | Minimum | Maximum |
| P4 | Fresh | Neat | 4.45[†] | 1.89 | 5.68 |
| | After Dilution | NC[1] | 3.30 | 1.78 | 3.99 |
| | | SP[2] | 0.94 | 0.00 | 1.65 |
| | | GTLS[3] | 0.00 | 0.00 | 1.09 |
| | After Freezing & Thawing | NC | 3.24 | 1.71 | 3.80 |
| | | SP | 1.02 | 0.00 | 1.86 |
| | | GTLS | 0.00 | 0.00 | 0.87 |
| JG1 | Fresh | Neat | 4.17 | 1.66 | 5.47 |
| | After Dilution | NC | 3.78 | 2.37 | 4.21 |
| | | SP | 1.10 | 0.00 | 1.85 |
| | | GTLS | 0.00 | 0.00 | 0.00 |
| | After Freezing & Thawing | NC | 2.32 | 1.41 | 3.45 |
| | | SP | 0.73 | 0.00 | 1.21 |
| | | GTLS | 0.00 | 0.00 | 0.94 |

[1]NC= Extender without antibiotics.
[2]SP=Streptomycin, 1000 µg/ml and penicillin, 1000 IU/ml.
[3]GTLS=Gentamycin, 500 µg/ml, tylosin, 100 µg/ml plus linco-spectin, 300/600 µg/ml.
[†]Values were transformed into log.

**Table 2.** Effect of antibiotics in semen extender on appearance of bacteria isolated at different stages of cryopreservation in buffalo bulls.

| Bull | Stage | Treatment | Isolates† (n) | Frequency of appearance (%) of bacterial isolates | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Staphylo-coccus | Micro-coccus | E. coli | Pseudo-monas | Proteus | Bacillus |
| P4 | Fresh | Neat | 21 | 22 | 12 | 27 | 9 | 11 | 20 |
| | After Dilution | NC[1] | 13 | 12 | 11 | 0 | 22 | 11 | 44 |
| | | SP[2] | 7 | 0 | 40 | 20 | 0 | 0 | 40 |
| | | GTLS[3] | 3 | 0 | 67 | 33 | 0 | 0 | 0 |
| | After Freezing & Thawing | NC | 8 | 33 | 0 | 17 | 33 | 0 | 17 |
| | | SP | 4 | 25 | 0 | 34 | 16 | 25 | 0 |
| | | GTLS | 1 | 0 | 0 | 100 | 0 | 0 | 0 |
| G1 | Fresh | Neat | 17 | 21 | 9 | 23 | 19 | 12 | 16 |
| | After Dilution | NC | 11 | 18 | 16 | 11 | 24 | 8 | 23 |
| | | SP | 5 | 33 | 12 | 0 | 34 | 5 | 16 |
| | | GTLS | 3 | 66 | 34 | 0 | 0 | 0 | 0 |
| | After Freezing & Thawing | NC | 9 | 0 | 20 | 12 | 30 | 15 | 23 |
| | | SP | 3 | 75 | 0 | 25 | 0 | 0 | 0 |
| | | GTLS | 1 | 0 | 100 | 0 | 0 | 0 | 0 |

[1]NC= Extender without antibiotics; [2]SP=Streptomycin, 1000 µg/ml and penicillin, 1000 IU/ml.
[3]GTLS=Gentamycin, 500 µg/ml, tylosin, 100 µg/ml plus linco-spectin, 300/600 µg/ml.
†Isolates represent the colonies, which appeared after 24 hours of incubation at 37°C.

**Table 3.** *In vitro* sensitivity of antibiotics to bacteria isolated from fresh semen samples of buffalo bulls.

| Organism | Isolates[†] (n) | Resistance (%) of isolates to antibiotics | | | | | |
|---|---|---|---|---|---|---|---|
| | | Streptomycin | Penicillin G | Gentamycin | Tylosin | Lincomycin | Spectinomycin |
| Staphylococcus | 5 | 0 | 90 | 0 | 20 | 0 | 15 |
| Micrococcus | 3 | 38 | 0 | 22 | 0 | 20 | 8 |
| *E. coli* | 7 | 40 | 88 | 33 | 0 | 0 | 0 |
| Pseudomonas | 3 | 80 | 100 | 34 | 77 | 0 | 0 |
| Proteus | 2 | 20 | 60 | 0 | 32 | 35 | 60 |
| Bacillus | 6 | 0 | 20 | 0 | 10 | 26 | 0 |

[†]Isolates represent the colonies, which appeared after 24 hours of incubation at 37°C.

## DISCUSSION

The present study determined the efficacy of a new antibiotic combination, GTLS, on TABC and appearance of bacteria isolated in fresh, diluted and frozen-thawed semen of buffalo bulls. Also *in vitro* sensitivity of GTLS to bacterial isolates from fresh semen was determined. Total aerobic bacterial counts, frequency of appearance of bacterial genera in semen samples at different stages of cryopreservation and sensitivity of bacterial isolates from fresh semen to antibiotics were measured as an indicator of bacterial control in this study. TABC was considerably lower in semen samples treated with GTLS than those of SP. Gentamycin and linco-spectin, being broad spectrum, are more effective against gram-positive and negative bacteria, and tylosin is more responsive against mycoplasmas (Ball et al., 1987; Shin et al., 1988). Another explanation could be that some of the organisms, due to excessive use of the drug, had mutated and became resistant (Alford, 1953; Parusov, 1974; Ahmad and Foote, 1985). Alternatively, the sensitivity of SP against the micro-organisms was reduced. The reduction in TABC in semen samples of NC compared to that of fresh in this study is most likely due to the dilution factor. The presence of TABC in frozen-thawed semen samples suggests that perhaps more stringent measures of hygenicity are required during processing. Alternatively the bacteria can even survive the deep-freezing and remain latent during storage at -196°C (Brown et al., 1974; Bielanski et al., 2003; Mazzilli et al., 2006).

The results on the frequency of appearance of bacterial genera demonstrated that fewer genera were present in samples treated with GTLS and their intensity was reduced compared to those of SP or NC. These observations fit well with the data on TABC of this study where efficacy of GTLS was unequivocal in restricting microbial growth. Our findings regarding the frequency of occurrence of aerobic bacteria in bovine semen are in agreement with the earlier studies (Salisbury et al., 1978; Naidu et al., 1982; Aleem et al., 1990). The predominance of pseudomonas and *E. coli* in buffalo semen could perhaps be linked to lowered fertility usually observed in buffaloes (Aleem et al., 1990; Din et al., 1990). The 48 hours old cultures from buffalo semen of these organisms, when injected to mice through intra-peritoneal route, killed them within 24 hours (Aleem et al., 1990). Wierzbowski et al. (1980) reported that pseudomonas and *E. coli* isolated from cattle bull semen had toxic effects on mouse embryo development. Incidentally, Parusov (1974) and Palli et al., (1975) reported that pseudomonas was resistant to SP.

The data on antibiotic sensitivity showed that the majority of the bacterial isolates from buffalo bull semen samples showed more sensitivity towards gentamycin, tylosin, lincomycin and spectinomycin than streptomycin and penicillin. The resistance of pseudomonas to streptomycin and penicillin were also reported by Parusov (1974), Palli et al. (1975) and Aleem et al. (1990). Gentamycin was 100% effective against proteus, while penicillin exhibited no inhibitory effect. Similar findings were reported by Overgoor (1967) and Aleem et al. (1990). Penicillin showed maximum resistance against staphylococcus which is in

agreement with the findings of Aleem et al. (1990). These findings were, however completely in disagreement with the results of Ahmed (1984) who reported 100% sensitivity of staphylococcus and proteus isolates to penicillin. It is reported that bacteria become resistant due to prolong use of same antibiotics for control of bacteriospermia (Alford, 1953; Parusov, 1974; Ahmad and Foote, 1985). Moreover it has been documented that some antibiotics have no or little effect against some specific isolates of buffalo bull semen (Aleem et al., 1990; Ali et al., 1994).

On the basis of these results it can, therefore, be hypothesized that GTLS which is more effective combination and improves the bacterial quality of semen will result in better semen quality and higher pregnancy rate in buffalo. In summary, the new antibiotic combination, GTLS, in extender was more sensitive to bacterial isolates from buffalo bull semen. Also GTLS compared to the conventional combination of antibiotics (SP) in semen extenders, resulted in considerable reduction of aerobic micro-organisms in post-thaw samples. Therefore, it can be concluded that GTLS efficiently controlled the bacteria in buffalo bull semen.

# CHAPTER 3

## EFFECT OF A NEW ANTIBIOTIC COMBINATION ON POST-THAW MOTILITIES, MOTION CHARACTERISTICS, MEMBRANE INTEGRITY, AND MORPHOLOGY OF BUFFALO BULL SPERMATOZOA

## ABSTRACT

This study determined the effect of a new antibiotic combination, i.e., gentamycin, tylosin and linco-spectin (GTLS) on post-thaw motilities, motion characteristics, plasma membrane integrity, and morphology (head, mid-piece, tail and acrosome) of buffalo bull spermatozoa. Semen was collected with artificial vagina from two healthy Nili-Ravi buffalo bulls under standard conditions. Split pooled ejaculates (n=5), possessing more than 60% sperm motility were diluted in Tris-citric acid (TCA) extender (at 37°C; $50\times10^6$ spermatozoa/ml), containing either GTLS (gentamycin 500 µg/ml, tylosin 100 µg/ml and linco-spectin 300/600 µg/ml), or streptomycin 1000 µg/ml and penicillin 1000 IU/ml (SP), or negative control with no antibiotics (NC). Samples were cooled to 4°C in 2 hours, equilibrated at 4°C for 4 hours, filled in 0.5 ml straws and frozen in a controlled rate cell freezer before plunging them into liquid nitrogen (-196°C). Deep-frozen semen was thawed at 37°C for 45 seconds. Post-thaw sperm motilities, motion characteristics, plasma membrane integrity and morphology (head, mid-piece, tail and acrosome) were determined. Mean motilities (visual, computer-assisted linear and circular), velocities (straight-line, average path and curvilinear) and amplitude of lateral head displacement in post-thaw semen samples did not differ ($P>0.05$) due to antibiotics. Similarly, sperm plasma membrane integrity did not vary ($P>0.05$). Morphologically abnormal spermatozoa (%) were lower ($P<0.05$) in GTLS ($17.7 \pm 2.8$) and SP ($16.9 \pm 3.1$) than in NC ($28.2 \pm 3.9$). Sperm cells possessing normal acrosome (%) were higher ($P<0.05$) in GTLS

(81.2 ± 3.1) and SP (79.9 ± 3.8) than in NC (72.1 ± 3.1). In conclusion, GTLS was not detrimental to post-thaw motilities, motion characteristics, membrane integrity and morphology of buffalo bull spermatozoa.

## INTRODUCTION

The main goal for artificial insemination (AI) in farm animals is to achieve genetic improvement. However transmission of bacteria by deep-frozen semen constitutes a risk to reproductive performance, which must be avoided. Microbial agents in semen used for AI, therefore, need to be effectively controlled by adding antibiotics in freezing diluents.

The addition of streptomycin and penicillin (SP) in the semen extender which has been historically used in AI industry was questioned as these antibiotics were not useful against corynebacterium, pseudomonas, vibrios, brucellae, mycobacterium, mycoplasmas, ureaplasmas and haemophilus (Roberts, 1971; Shin et al., 1988). Moreover, some of the organisms which were previously sensitive to SP became resistant to these antibiotics (Alford, 1953; Parusov, 1974; Ahmad and Foote, 1985). This necessitated the screening of wide array of antibiotics for their effects on semen quality and fertility in bovine (Ahmad and Foote, 1985; Ahmad and Foote, 1986; Ahmad et al., 1987). Later, some of the selected antibiotics were systematically studied, for their dose, method of addition and interaction with the extender and their effects on microbes (Shin et al., 1986; Shin et al., 1988). This led to the development of a new antibiotic combination (gentamycin, tylosin and linco-spectin; GTLS) for cryopreservation of cattle bull semen (Doak, 1986). This new combination of antibiotics was most effective in controlling micro-organisms present in bovine semen (Shin et al., 1988) and was not detrimental to semen quality (Lorton et

al., 1988a; Ericsson et al., 1990; Guerin and Thibier, 1993) or fertility (Lorton et al., 1988b; Sullivan et al., 1988; Kupferschmied et al., 1991a,b). At present, this combination of new antibiotics for bull semen is commonly used in commercial and research centres related to AI in the developed countries (Certified Semen Services (CSS®), 2002).

There are reports providing information on occurrence of pathogenic and non-pathogenic bacteria in buffalo bull semen (Naidu et al., 1982; Pal et al., 1989; Aleem et al., 1990; Pal et al., 1993; Bindra et al., 1994; Sharma et al., 1996; Amin et al., 1999). However, few scattered studies are available on effect of ampicillin, kanamycin or gentamycin compared to conventional antibiotics (SP) in extender on quality of buffalo spermatozoa (Hussain et al., 1990; Ali et al., 1994). Moreover, isolated studies suggest that conventional antibiotics (SP), when replaced with ampicillin, kanamycin or gentamycin were non-toxic to buffalo bull spermatozoa (Hussain et al., 1990; Ali et al., 1994). On the basis of previous information we hypothesized that the new combination of antibiotics (GTLS) in the semen extender should not be detrimental to the buffalo bull spermatozoa. This study was primarily designed to determine the effect of a new antibiotic combination, GTLS, on post-thaw motilities, motion characteristics, plasma membrane integrity, general and acrosomal morphology of buffalo bull spermatozoa.

## Preparation of extenders

Tris-citric acid (TCA) was used as the buffer for the experimental extenders. It consisted of 1.56 gm citric acid (Fluka, Switzerland) and 3.0 gm tris–(hydroxymethyl)-aminomethane (Sigma, St. Louis, MO) in 74 ml distilled water. The pH of buffer was 6.8 and the osmotic pressure was 320 mOsmol/Kg. Egg yolk (20%; vol/vol), fructose (0.2%; wt/vol; Merck, F.R Germany) and glycerol (6%; vol/vol; Merck, F.R Germany) were added to each of the three experimental extenders.

First extender was a combination of four antibiotics (GTLS). It comprised of gentamycin available as gentamycin sulphate (Gibco, Madison, WI) 561 µg/mg, which was added at rate of 500 µg/ml, tylosin tartrate (Elanco, Indianapolis, Indiana) was added at the rate of 100 µg/ml and linco-spectin commercially available as lincomycin hydrochloride (Upjohn Co, Kalamazoo, MI) 50 mg/ml, and spectinomycin sulphate (Upjohn Co, Kalamazoo, MI) 100mg/ml, added at the rate of 300/600 µg/ml (Shin et al., 1988). Second extender (SP) contained streptomycin (Sigma, St. Louis, MO) available as streptomycin sulphate 761 IU/mg added at the rate of 1000 µg/ml and penicillin (Antibiotics, Mianwali, Pakistan) available as benzyl penicillin 500,000 IU added at the rate of 1000 IU/ml. Third extender (NC) did not contain antibiotics and served as negative control.

**Semen collection and evaluation**

Semen was collected from two healthy Nili-Ravi buffalo bulls (P4 and JG1) maintained at Livestock Research Station, National Agricultural Research Centre, Islamabad, Pakistan. Semen was collected in artificial vagina at 42°C at weekly intervals for a period of five weeks. The frequency of collection from each bull was two consecutive ejaculates per week. Each ejaculate was transferred to the laboratory within a minute and visual motility was assessed by using phase contrast microscope (400X; Olympus BX 40) attached with a closed circuit television. Sperm concentration was assessed by digital photometer (Dr. Lange LP 300 SDM, Germany). The qualifying ejaculates were pooled in order to have sufficient semen for a replicate and to eliminate the bull effect. The semen was given a holding time of 15 minutes at 37°C in water bath before dilution.

**Semen processing**

Three aliquots of semen were diluted at 37°C in a single step with one of the three experimental extenders in order to contain approximately $50\times10^6$ spermatozoa/ml. After dilution, the semen was cooled to 4°C in 2 hours @ 0.275 °C/minute and equilibrated for 4 hours at 4°C. Semen was then filled in 0.5 ml straws with suction pump at 4°C in the cold cabinet unit (Minitub, Germany) and frozen in programmable cell freezer (KRYO 10 series III, UK) from 4°C to -15°C at the rate of 3°C/minute and from -15°C to -80°C at the rate of 10°C/minute. Straws were then plunged into liquid nitrogen (-196°C)

and stored. After 24 hours, semen straws were thawed at 37°C for at least 45 seconds for assessment of post-thaw semen quality.

**Semen assays**

Visual motility assessment

A drop of semen was placed on a pre-warmed glass slide and cover-slipped. Visual motility of spermatozoa was assessed under microscope (400X) at 37°C.

Computer-assisted motilities and motion characteristics

A computer-assisted semen analyzer (CASA; SM-CMA version 4.4; Mika Medical GmbH, Germany) was used for precise quantification of sperm motilities and motion characteristics as recently described in buffalo bulls (Rasul et al., 2000). After thawing, a drop of semen was placed on Makler chamber (Sefi-Medical Industries, Haifa, Israel) having depth of 10 μm and was analyzed for motility (%), linear motility (%), circular motility (%), straight-line velocity (μm/second), average path velocity (μm/second), curvilinear velocity (μm/second) and amplitude of lateral head displacement (ALH; μm).

Plasma membrane integrity

Sperm plasma membrane integrity was assessed by hyposmotic swelling (HOS) assay as described earlier (Jeyendran et al., 1984). The solution of HOS contained sodium citrate (Merck, F.R Germany) 0.73 gm and fructose 1.35 gm, dissolved in 100 ml distilled water (osmotic pressure ~190 mOsmol/Kg),

according to the standards of the WHO (WHO, 1992). The assay was performed by mixing 50 µl of frozen-thawed semen sample to 500 µl of HOS solution and incubated at 37°C for 30 minutes. After incubation, a drop of semen sample was examined under phase contrast microscope (400X). One hundred spermatozoa were counted for their swelling characterized by coiled tail indicating intact plasma membrane.

Sperm morphology

Semen sample (0.5 ml), after thawing, was fixed in 50 µl of 1% formal citrate (2.9 gm trisodium citrate dihydrate; Merck, F.R Germany and 1 ml of 37% solution of formaldehyde; Merck, F.R Germany dissolved in 100 ml of distilled water). Morphological abnormalities (tail, mid piece and head) and acrosomal integrity characterized by normal apical ridge (NAR) of one hundred spermatozoa were assessed using phase contrast microscope (Leica, Leitz Wetzlar, Germany; 1000X) under oil immersion.

**Statistical analysis**

Results are presented as means ± SEM. Effect of antibiotics for different variables was analyzed by the analysis of variance (ANOVA). When the F–ratio was significant ($P<0.05$), Tukey's Honestly Significant Difference was used, to compare the treatment means (MINITAB® Release 12.22, 1998).

# RESULTS

## Visual and computer-assisted motilities

The data on effect of antibiotics on post-thaw motilities of buffalo bull spermatozoa are presented in Table 1. The overall means for visual motility (46.5 ± 4.2), computer-assisted motility (52.9 ± 2.8), linear motility (46.3 ± 9.2) and circular motility (24.0 ± 6.9) did not vary due to antibiotics.

## Motion characteristics

The data on effect of antibiotics on post-thaw velocities and ALH of spermatozoa in buffalo bull is presented in Table 2. The overall means for straight line velocity (41.5 ± 7.0 µm/second), average path velocity (54.3 ± 3.2 µm/second), curvilinear velocity (86.9 ± 5.5 µm/second) and ALH (4.5 ± 0.3 µm) of spermatozoa did not vary due to antibiotics.

## Membrane integrity and morphology

The effect of antibiotics on post-thaw membrane integrity of buffalo bull spermatozoa is presented in Figure 1. Plasma membrane integrity (%) of spermatozoa did not vary ($P>0.05$) due to antibiotics and the overall average was 56.8 ± 8.9.

The effect of antibiotics on post-thaw morphological abnormalities (head, mid-piece and tail) of buffalo bull spermatozoa is presented in Figure 2. The morphological abnormalities did not differ ($P>0.05$) between SP ($16.9 \pm 3.1$) and GTLS ($17.7 \pm 2.8$), but were higher ($P<0.05$) in NC ($28.2 \pm 3.9$).

The effect of antibiotics on post-thaw acrosome morphology (intact acrosome) of buffalo bull spermatozoa is presented in Figure 3. The percentage for normal acrosome did not differ between SP ($79.9 \pm 3.8$) and GTLS ($81.2 \pm 3.1$), however, they were lower ($P<0.05$) in NC ($72.1 \pm 3.1$).

**Table 1.** Effect of antibiotics in extender on post-thaw motilities (%) of buffalo bull spermatozoa.

| Variables | NC[1] | SP[2] | GTLS[3] |
|---|---|---|---|
| Visual motility | 39.0 ± 6.1[†] | 48.3 ± 3.7 | 52.2 ± 2.9 |
| Computer motility | 44.5 ± 3.8 | 55.6 ± 2.1 | 58.5 ± 2.5 |
| Linear motility | 40.2 ± 13.5 | 51.4 ± 6.1 | 47.3 ± 8.1 |
| Circular motility | 21.3 ± 7.9 | 28.0 ± 4.9 | 22.9 ± 7.9 |

[†] Values are (Mean ± SE) based on five replicates.
[1] NC=Extender without antibiotics.
[2] SP=Streptomycin, 1000 µg/ml and penicillin, 1000 IU/ml.
[3] GTLS=Gentamycin, 500 µg/ml, tylosin, 100 µg/ml plus linco-spectin, 300/600 µg/ml.
Means did not differ (P>0.05) due to antibiotics.

**Table 2.** Effect of antibiotics in extender on post-thaw velocities (μm/second) and amplitude of lateral head displacement (μm) of buffalo bull spermatozoa.

| Variables | NC[1] | SP[2] | GTLS[3] |
|---|---|---|---|
| Straight line velocity | 37.9[†] ± 9.9 | 41.4 ± 6.7 | 45.2 ± 4.4 |
| Average path velocity | 52.6 ± 2.8 | 56.6 ± 3.5 | 53.9 ± 3.1 |
| Curvilinear velocity | 87.3 ± 6.6 | 86.5 ± 3.9 | 86.9 ± 6.1 |
| Lateral head displacement | 4.8 ± 0.2 | 4.6 ± 0.2 | 4.2 ± 0.5 |

[†] Values are (Mean ± SE) based on five replicates.
[1] NC=Extender without antibiotics.
[2] SP=Streptomycin, 1000 μg/ml and penicillin, 1000 IU/ml.
[3] GTLS=Gentamycin, 500 μg/ml, tylosin, 100 μg/ml plus linco-spectin, 300/600 μg/ml.
Means did not differ (P>0.05) due to antibiotics.

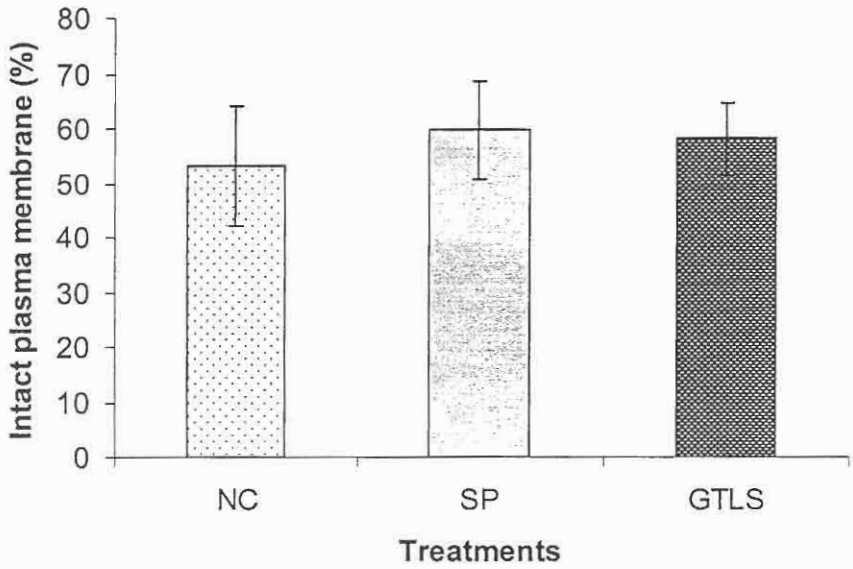


**Figure 1.** Effect of antibiotics (NC: extender without antibiotics; SP: streptomycin, 1000 µg/ml + penicillin, 1000 IU/ml; GTLS: gentamycin, 500 µg/ml + tylosin, 100 µg/ml + linco-spectin, 300/600) in extender on post-thaw membrane integrity (%) in buffalo bull spermatozoa. Values are (Mean ± SE) based on five replicates. Means did not differ (P>0.05) due to antibiotics.

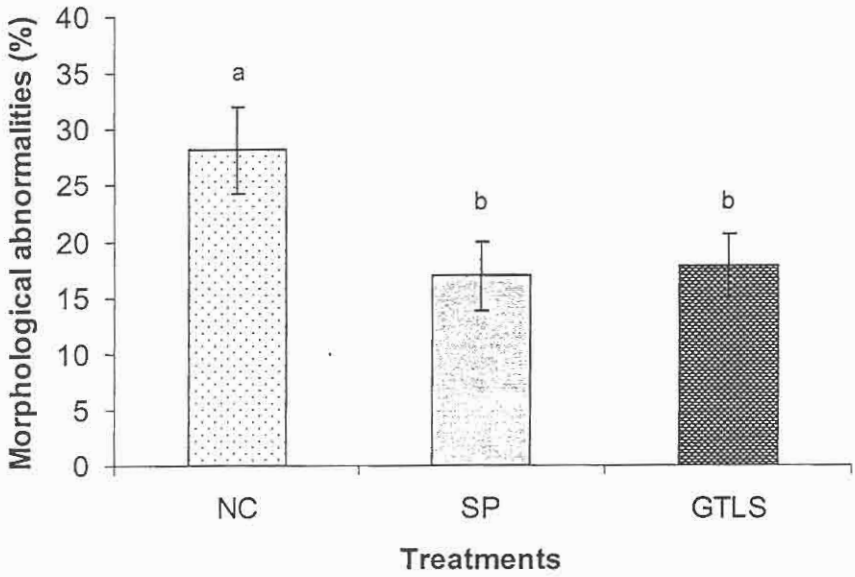


**Figure 2.** Effect of antibiotics (NC: extender without antibiotics; SP: streptomycin, 1000 µg/ml + penicillin, 1000 IU/ml; GTLS: gentamycin, 500 µg/ml + tylosin, 100 µg/ml + linco-spectin, 300/600) in extender on post-thaw morphological abnormalities (%) in buffalo bull spermatozoa. Values are (Mean ± SE) based on five replicates. Means with different superscripts differed ($P<0.05$) due to antibiotic.

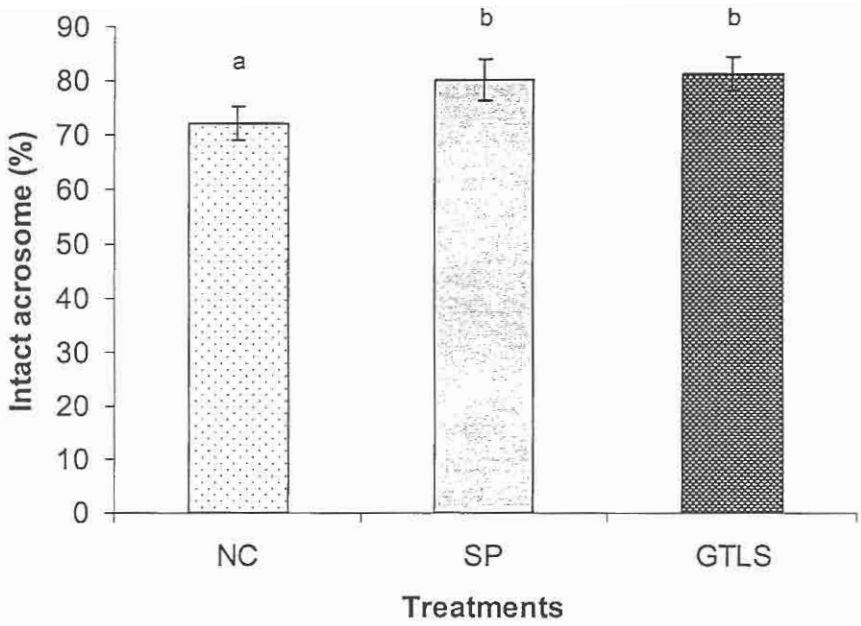


**Figure 3.** Effect of antibiotics (NC: extender without antibiotics; SP: streptomycin, 1000 µg/ml + penicillin, 1000 IU/ml; GTLS: gentamycin, 500 µg/ml + tylosin, 100 µg/ml + linco-spectin, 300/600) in extender on post-thaw intact acrosome (%) in buffalo bull spermatozoa. Values are (Mean ± SE) based on five replicates. Means with different superscripts differed ($P<0.05$) due to antibiotic.

## DISCUSSION

This study determined if GTLS in extender is detrimental to post-thaw motilities, motion characteristics, plasma membrane integrity, and morphology (head, mid-piece, tail and acrosome) of buffalo bull spermatozoa.

The pattern of sperm cell movement is sensitive to the chemical and physical properties of the medium in which they are suspended (Rasul et al., 2000). The motilities (visual, computer-assisted circular and linear) did not differ due to antibiotics in this study. These results are similar to those of Lorton et al. (1988a) who observed that GTLS had no adverse affect on post-thaw progressive motility of bovine spermatozoa tested at three commercial AI centres. Percentage of post-thaw motile spermatozoa declined when concentration of gentamycin exceeded 500 µg/ml in milk and 1500 µg/ml in TCA extender (Ahmad and Foote, 1985). In an earlier study on buffalo, kanamycin (500 µg/ml) or ampicillin (250 µg/ml), tested individually did not affect the motility or liveability of spermatozoa in samples after 24 hours of storage (Hussain et al., 1990). Similarly, ampicillin (250 µg/ml) was found to be relatively better on motility and survival of buffalo spermatozoa than streptomycin and penicillin or gentamycin sulphate used in lower dose (Ali et al., 1994). Previously, kanamycin, erythromycin, streptomycin and terramycin were shown to be deleterious to mammalian spermatozoa, whereas gentamycin and lincomycin had no detrimental effects (Stallcup and

McCartney, 1953; Back et al., 1975). Loss in motion characteristics of stallion spermatozoa due to increase in dose of antibiotics in extender has also been reported (Jasko et al., 1993). Mechanisms by which most of the antibiotics adversely affect sperm viability are through inhibition of protein synthesis, impairment of folic acid synthesis and intercalation with DNA (Gilman et al., 1985). Thus, GTLS in combination appears to be equally good as an alternate for cryopreservation of buffalo bull spermatozoa.

The measurement of velocity has been considered as an indirect indicator of mitochondrial function of a spermatozoon (Graham et al., 1984) and is associated with fertility (Budworth et al., 1988). Amplitude of lateral head displacement may indirectly reflects the efficiency of the flagellar beat pattern, appears to be a critical factor whenever the generation of shearing forces is involved, as in sperm penetration of cervical mucus or their ability to penetrate the cumulus oophorus and the zona pellucida (Jeulin et al., 1986). Sperm motion characteristics, in this study, demonstrated that the mean velocities (straight-line, average path and curvilinear) and ALH did not differ due to antibiotics. These values are in general agreement with a previous study from our laboratory in which TCA buffering system containing conventional antibiotics (SP) was used for buffalo spermatozoa (Rasul et al., 2000). Indirectly, this suggests that GTLS preserved the mitochondrial and other cellular functions of the spermatozoa well. Amikacin sulphate and potassium penicillin most commonly sustained sperm motion characteristics in cooled equine semen (Varner, 1991). However, the speed of the sperm movement

was affected adversely with increasing minocin concentration (Ahmad and Foote, 1984). Because the GTLS did not change the curvilinear velocity and ALH, motion characteristics associated with sperm capacitation and acrosome reaction remained the same.

The evaluation of plasma membrane integrity is of particular importance due to its involvement in metabolic exchanges with the surrounding medium. Furthermore, the process of capacitation, acrosome reaction and the oocyte penetration requires a biochemically active membrane (Jeyendran et al., 1984). Plasma membrane functional integrity of buffalo spermatozoa was evaluated using HOS assay. The swelling ability of frozen-thawed spermatozoa in this experiment did not vary due to antibiotics, suggesting that GTLS is not detrimental to plasma membrane integrity. Overall percentage was higher compared to those of Rasul et al., (2000) who used SP in freezing diluents. It is relevant to mention that gram-negative micro-organisms are reported to attach themselves to spermatozoal membranes (Bolton et al., 1986; Wolff et al., 1993). Bonadonna (1971) and Diemer et al. (1996) reported that binding of the bacteria resulted in micro-structural damage to sperm plasmalemma.

Morphological defects of spermatozoa, which have been associated with reduced fertility in bovine with AI, are abnormal heads, coiled tails and proximal droplets (Linford et al., 1976, Wood et al., 1986, Soderquist et al., 1991). According to Graham (1996), poor semen handling techniques or sub-optimal freezing conditions may induce irreversible morphological changes

such as impulse of the sperm tail. In the present study, morphological abnormalities were less in case of GTLS and SP than NC.

The presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is being required at the proper time to facilitate fertilization (Thomas et al., 1997). The change in acrosomal cap is mainly due to sperm injury or aging (Saacke and Marshall, 1968). In the present study, the sperm cells possessing normal acrosome were higher in case of GTLS and SP than NC. In a similar pattern, acrosomal integrity was not significantly affected with GTLS compared to the combination of penicillin, dihydrostreptomycin and polymyxin B sulphate (Lorton et al., 1988a). Findings of Gerard et al. (1995) are also in agreement with the present study, who reported that addition of GTLS to bovine semen extender, significantly improved intact acrosome as compared with the control without antibiotics. Damage to acrosome in NC may be due to bacterial growth. Presence of micro-organisms, especially the bacteria in the ejaculates can affect fertilization directly (Morrell, 2006), by inducing acrosome reaction (El-Mulla et al., 1996). Ahmad and Foote (1985) found that acrosomal integrity was increased with addition of gentamycin as compared to SP. Based on these observations, it appears, as if the events leading to cellular injury are of progressive in nature and changes first occur in the acrosomal morphology.

In summary, GTLS, in freezing diluent compared to the conventional antibiotic combination (SP) did not differ in post-thaw motilities, motion characteristics,

plasma membrane integrity, morphology (head, mid-piece, tail and acrosome) of buffalo bull spermatozoa. Therefore, it can be concluded that GTLS is not detrimental to semen quality of buffalo bull.

# CHAPTER 4

# EFFECT OF A NEW ANTIBIOTIC COMBINATION ON FERTILITY OF FROZEN BUFFALO BULL SEMEN

## ABSTRACT

This study was carried out to determine if a new antibiotic combination comprising of gentamycin, tylosin and linco-spectin (GTLS) in extender is suitable for improvement in fertility of frozen buffalo bull semen to obtain better pregnancy rate through artificial insemination (AI). Semen was collected by using an artificial vagina (42°C) at weekly intervals in two batches from a healthy Nili-Ravi buffalo bull of known fertility. The ejaculates were split-sampled and diluted with Tris-citric acid extender (at 37°C; $50\times10^6$ spermatozoa/ml), containing either SP (streptomycin 1000 µg/ml and penicillin 1000 IU/ml) or GTLS (gentamycin 500 µg/ml, tylosin 100 µg/ml and linco-spectin 300/600 µg/ml). Fertility test based on 75-days first service pregnancy rate was determined under field conditions. A total of 650 buffaloes were inseminated with experimental frozen semen. Out of these 591 animals could be followed, including 292 of SP and 299 of GTLS group. The inseminations were performed in two batches and each batch spread over a period of three months. The data on fertility were compared by using chi-square statistics. The fertility rates for SP-based vs. GTLS-containing frozen semen of buffalo bull were 42.8 and 55.2%, respectively. The results for GTLS were significantly higher than SP. The fertility rates also differed significantly in the first and second batch of inseminations performed with SP or GTLS-based cryopreserved semen of buffalo bull. In conclusion, seminal quality measured by field fertility trial indicated that GTLS combination of antibiotics added to the semen extender was better for improvement in the

fertility of frozen buffalo bull semen, by yielding better pregnancy rates through AI.

## INTRODUCTION

The main purpose of preserving semen is to maintain the fertilizing capacity of spermatozoa while diluting the ejaculate to maximise the use of superior genetic bulls (Kommisrud et al., 1996). Moreover, the risk of spreading pathogens through semen needs to be effectively reduced (Kapoor, 2003). The presence of micro-organisms, especially the bacteria in the ejaculates can affect fertilization directly (Morrell, 2006), by adhering to spermatozoa (Wolff et al., 1993; Diemer et al., 1996), impairing their motility (Panangala et al., 1981; 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).

Addition of antibiotics to semen extender was one of the first major advances to significantly impact the fertility potential of artificial insemination (AI) in bovine (de Jarnette et al., 2004). Recent published worldwide statistical data revealed that approximately 264 million doses of bovine semen were cryopreserved for commercial use in the year 2002 (Thibier and Wagner, 2002). In Pakistan the average annual production of cryopreserved bovine semen for last five years is more than one million (Ullah and Andrabi, 2005). Traditionally, streptomycin and penicillin (SP) is the antibiotic combination that has been added to the freezing diluents for buffalo bull semen (Sansone et al., 2000). However, current international standards (Certified Semen Services (CSS®), 2002) with regard to the antibiotic components of semen

extenders have made it necessary to look for alternatives for the SP-containing extender for buffalo bull semen cryopreservation.

It has been demonstrated that the combination of gentamycin, tylosin and linco-spectin (GTLS) is more effective for controlling micro-organisms including mycoplasmas, ureaplasmas, *Campylobacter fetus*, *Haemophilus somnus*, and pseudomonas in bovine semen than other antibiotics or combination of antibiotics added to extenders (Shin et al., 1986; Shin et al., 1988; Guerin and Thibier, 1993). These micro-organisms can cause infection in the reproductive tract leading to abortion, lowered fertility or infertility (Friberg, 1980; Truscott, 1981; Humphrey et al., 1982; Truscott and Brown, 1986; Eaglesome and Garcia, 1990; Eaglesome et al., 1995; Cardoso et al., 2000; Sylla et al., 2005). Also 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 (Lorton et al., 1988a,b, Sullivan et al., 1988; Ericsson et al., 1990; Kupferschmied et al., 1991a,b; Gerard et al., 1995; Bousseau et al., 1998).

Effect of the relatively new antibiotic combination (GTLS) in semen extender on fertility has been widely assessed in cattle (Lorton et al., 1988b; Sullivan et al., 1988; Kupferschmied et al., 1991a,b; Kommisrud et al., 1996; Bousseau et al., 1998), while no information is available in case of water buffalo. The present study was conducted to determine the suitability of GTLS in extender

for improvement in fertility of deep-frozen buffalo bull spermatozoa to obtain better pregnancy rate through AI under field conditions.

## Semen collection and evaluation

Semen was collected by using an artificial vagina (42°C) at weekly intervals in two batches from a healthy Nili-Ravi buffalo bull of known fertility (P4; *Bubalus bubalis*). The bull was maintained at Livestock Research Station, National Agricultural Research Centre, Islamabad. Each batch of collection was spread over a period of five weeks. The frequency of semen collection was two consecutive ejaculates per week. Immediately after collection visual motility of each ejaculate was assessed by using phase contrast microscope (400X) attached with a closed circuit television. Sperm concentration was assessed by digital spectrophotometer (Dr. Lange LP 300 SDM, Germany) at 560 nm. Semen samples possessing more than 60% motility and $500x10^6$ spermatozoa/ml were used. A total of six ejaculates were used in first batch, whereas a total of seven ejaculates were used in second batch for further processing. The semen was given a holding time of 10 to 15 minutes at 37°C in water bath before dilution

## Dilution with Tris-citric acid extender

Tris-citric acid (TCA) was used as the buffer for the experimental extenders. It consisted of 1.56 gm citric acid (Fluka, Switzerland) and 3.0 gm tris–(hydroxymethyl)-aminomethane (Sigma, St. Louis, MO) in 74 ml distilled

water. The pH of buffer was 6.8 and the osmotic pressure was 320 mOsmol/Kg. Egg yolk (20%; vol/vol), fructose (0.2%; wt/vol; Merck, F.R Germany) and glycerol (6%; vol/vol; Merck, F.R Germany) were added to each of the two experimental extenders.

First extender (SP) contained streptomycin (Sigma, St. Louis, MO) available as streptomycin sulphate 761 IU/mg added at the rate of 1000 µg/ml and penicillin (Antibiotics, Mianwali, Pakistan) available as benzyl penicillin 500,000 IU added at the rate of 1000 IU/ml. Second extender was a combination of four antibiotics (GTLS). It comprised of gentamycin available as gentamycin sulphate (Gibco, Madison, WI) 561 µg/mg, which was added at rate of 500 µg/ml, tylosin tartrate (Elanco, Indianapolis, Indiana) was added at the rate of 100 µg/ml and linco-spectin available as lincomycin hydrochloride (Upjohn Co, Kalamazoo, MI) 50 mg/ml, and spectinomycin sulphate (Upjohn Co, Kalamazoo, MI) 100mg/ml, added at the rate of 300/600 µg/ml (Shin et al., 1988).

**Semen processing**

The ejaculates were split and diluted at 37°C in a single step with one of the two experimental extenders in order to contain approximately $50\times10^6$ motile spermatozoa/ml. After dilution, the semen was cooled to 4°C in 2 hours @ 0.275 °C/minute and equilibrated for 4 hours at 4°C. Semen was then filled in 0.5 ml straws with suction pump at 4°C in the cold cabinet unit (Minitub,

Germany) and frozen in programmable cell freezer (KRYO 10 series III, UK) from 4°C to -15°C at the rate of 3°C/minute and from -15°C to -80°C at the rate of 10°C/minute. Straws were then plunged into liquid nitrogen (-196°C) and stored.

**Semen quality control and artificial insemination**

After 24 hours of storage in liquid nitrogen (-196°C), post-thaw seminal quality (visual motility) was assured by routine procedure as described in previous chapter.

A total of 650 buffaloes with clinically normal reproductive tract and showing signs of true oestrus were inseminated with experimental frozen semen in Tehsil Hazro, District Attock and Tehsil Kahore Pacca, District Lodhran under field conditions. Out of these 591 animals could be followed, including 292 of SP group and 299 of GTLS group. All the experimental inseminations were performed approximately 24 hours after onset of heat. The artificially bred animals were examined for pregnancy through rectal palpation at least 75 days post-insemination. The inseminations were performed in two batches and each batch spread over a period of three months.

**Statistical analysis**

The data on fertility rate were compared by using chi-square statistics

(MINITAB® Release 12.22, 1998). A $P<0.05$ was considered significant.

## RESULTS

### Fertility rates

The data on effect of two combinations of antibiotics i.e. SP or GTLS, added to extender on fertility of frozen buffalo bull spermatozoa is presented in Table 1. The fertility rates for SP-based vs. GTLS-containing frozen semen of buffalo bull were 42.8 and 55.2%, respectively. The results for GTLS were significantly higher than that of SP.

The effect of two combinations of antibiotics (SP or GTLS) in semen extender on fertility of cryopreserved buffalo bull spermatozoa in relation to batch of inseminations performed is presented in Table 2. The fertility rates differed significantly in the first and second batch of inseminations performed with SP or GTLS-based cryopreserved semen of buffalo bull. The results for GTLS were significantly higher than that of SP.

**Table 1.** Effect of antibiotics in semen extender on fertility rate (%) of cryopreserved buffalo bull spermatozoa.

| Antibiotics | No. of inseminations performed | *No. of pregnancy test performed | Pregnancies achieved | Chi-square | P-value |
|---|---|---|---|---|---|
| SP[1] | 325 | 292 | 125 (42.80%) | 9.054 | 0.003 |
| GTLS[2] | 325 | 299 | 165 (55.18%) | | |

[1]SP=streptomycin, 1000 µg/ml and penicillin, 1000 IU/ml.
[2]GTLS=gentamycin, 500 µg/ml, tylosin, 100 µg/ml plus linco-spectin, 300/600 µg/ml.
*Some inseminated animals could not be tested for pregnancy for various reasons.

**Table 2.** Effect of antibiotics in semen extender on fertility rate (%) of cryopreserved buffalo bull spermatozoa in relation to batch of inseminations performed.

| Batch No. | Antibiotics | No. of inseminations performed | *No. of pregnancy test performed | Pregnancies achieved | Chi-square | P-valu |
|---|---|---|---|---|---|---|
| 1 | SP[1] | 100 | 91 | 38 (41.75%) | 3.807 | 0.05 |
| | GTLS[2] | 100 | 85 | 48 (56.47%) | | |
| 2 | SP | 225 | 201 | 87 (43.28%) | 5.379 | 0.02 |
| | GTLS | 225 | 214 | 117 (54.67%) | | |

[1]SP=streptomycin, 1000 µg/ml and penicillin, 1000 IU/ml.
[2]GTLS=gentamycin, 500 µg/ml, tylosin, 100 µg/ml plus linco-spectin, 300/600 µg/ml.
*Some inseminated animals could not be tested for pregnancy for various reasons.

## DISCUSSION

The findings of present experiment are in line with previous studies which have shown that GTLS as a component of various semen extenders had no negative influence on pregnancy or non-return (NR) rates in bovine when compared with other antibiotics used alone or in combinations. Stoyanov (1987) reported that gentamycin is best antibiotic for bull semen extender as compared to penicillin or streptomycin in terms of higher fertility rates. Whereas, Lorton et al (1988b) and Sullivan et al. (1988) reported no significant effect on seminal quality as measured by field fertility using GTLS or dihydrostreptomycin, penicillin and polymyxin B sulphate with or without linco-spectin in heated whole-milk or egg yolk-sodium citrate extenders. More recently, Bousseau et al. (1998) reported a similar trend for *in vitro* and *in vivo* fertility tests conducted with GTLS-based Biociphos plus®extender and Laiciphos® extender containing SP plus linco-spectin. The significant difference in fertility rate in present study can be attributed to the use of only two antibiotics in combination (SP) vs. GTLS.

Kupferschmied et al. (1991a) found no difference in fertility rate in cows on using SP or GTL with or without spectinomycin in Tris-diluents prior to deep freezing. Kupferschmied et al. (1991b) also reported no significant difference in NR rates in cows inseminated with frozen-thawed semen containing either SP or GTLS. Better efficacy of GTLS in the present study could be due to difference in method of addition of antibiotics in semen diluent. Also in current

study, the lowered fertility rates in buffaloes observed with SP 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 SP (Aleem et al., 1990). Results of Shin et al. (1988) have indicated that GTLS has a broader spectrum of microbial control in frozen bovine semen than SP with or without polymyxin B. Thus, 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 seminal quality/fertility (Lorton et al., 1988b). Influence of GTLS has also been compared after *in vitro* fertilisation and culture of bovine embryos, with higher cleavage rate for GTLS-containing Biladyl® (TCA) extender (Lonergan et al., 1994). In contrast to our findings, Kommisrud et al. (1996) reported significantly higher NR rates with SP-containing skim milk-egg yolk extender as compared to GTLS-based Biladyl® extender in cattle. This variation may possible be attributed to the difference in semen extenders and dilution methods. Other then semen quality, the fertility rates are affected by a number of other factors including female reproductive status and genetic, management and nutrition (Younis et al., 1999; Graham and Moce, 2005). Also this variation might be due to technical know how, season and geographical area (Rodriguez-Martinez, 2003).

In summary, the new antibiotic combination, GTLS, in semen extender compared to the conventional antibiotic combination, SP, resulted in significantly improving the fertility of frozen buffalo bull semen, by obtaining

better pregnancy rate through AI. In conclusion, seminal quality measured by field fertility trial indicates that GTLS combination of antibiotics is more suitable in semen extender for improvement in the fertility of frozen buffalo bull semen.

## GENERAL DISCUSSION

The isolation of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa, Pseudomonas putida*, *Pseudomonas testosteroni* and *Proteus mirabilis* in the present study is significant because of their deleterious effects on viability of buffalo bull spermatozoa as has been reported by other workers (Naidu et al., 1982; Aleem et al., 1990; Ramaswamy et al., 1997; Shukla et al., 2005). Bacteria appear to exert their spermicidal effect directly upon the sperm cell (Althouse and Lu, 2005).

The predominance of *Staphylococcus aureus* (16%), *Escherichia coli* (16%) and *Pseudomonas aeruginosa* (13%) in present study is of great significance because of their negative impact on vitality of spermatozoa (Kapoor, 2003). *Staphylococcus aureus* is reported to alter sperm function *in vitro* by interfering with the metabolic processes (Borcyckzo, 1982). Interaction of sperm with *Escherichia coli*, a contaminant in human semen, has been one of the most extensively studied (Auroux et al., 1991; Wolff et al., 1993; Monga and Roberts, 1994; Diemer et al., 1996). Wolff et al. (1993) elucidated that *Escherichia coli* adhered to sperm surface via mannose binding sites. Diemer et al. (1996) reported that binding of the *Escherichia coli* resulted in ultrastructural damage to sperm plasma membrane. Huwe et al. (1998) reported that *Escherichia coli* lowered sperm motility *in vitro*. *Escherichia coli* is also associated with uterine infections and altered fertility in cows (Griffin et

al., 1974a,b; Del Vecchio et al., 1994; Dhaliwal et al., 2001; Foldi et al., 2006). *Pseudomonas aeruginosa* can cause serious genital diseases in breeding bulls (Borcyckzo, 1982) and interfere with the sperm cell viability and probably lower the conception rate too (Eaglesome and Garcia, 1995; Corona et al., 2006). Also if *Pseudomonas aeruginosa* is transmitted to the female's genital tract at the time of breeding, it can result in infectious endometritis and associated subfertility (Varner et al., 1998). Aleem et al. (1990) reported that the 48 hours old cultures from buffalo semen of *Pseudomonas aeruginosa*, when injected to mice through intra-peritoneal route, killed them within 24 hours. The isolation of *Proteus mirabilis* in present study from buffalo bull semen is also of great significance, because it is often implied in infertility cases of equines (Clement et al., 1993). It is also suggested that the kinds of bacteria found in semen under a given set of circumstances depends on the microbial population available to invade the genital tract (and also, therefore, on management of bulls) as well as on the ability of bacteria to survive conditions in the preputial cavity and urethra (Brown et al., 1974).

Total aerobic bacterial counts (TABC), frequency of appearance of bacterial genera in semen samples at different stages of cryopreservation and sensitivity of bacterial isolates from fresh semen to antibiotics were measured as an indicator of bacterial control in this study. TABC was considerably lower in semen samples treated with GTLS (gentamycin 500 µg/ml, tylosin 100 µg/ml and linco-spectin 300/600 µg/ml) than those of SP (streptomycin 1000 µg/ml and penicillin G 1000 IU/ml). Gentamycin and linco-spectin, being broad spectrum, are more

effective against gram-positive and negative bacteria, and tylosin is more responsive against mycoplasmas (Ball et al., 1987; Shin et al., 1988). Another explanation could be that some of the organisms, due to excessive use of the drug, had mutated and became resistant (Alford, 1953; Parusov, 1974; Ahmad and Foote, 1985). Alternatively, the sensitivity of SP against the micro-organisms was reduced. The reduction in TABC in semen samples of NC compared to that of fresh in this study is most likely due to the dilution factor. The presence of TABC in frozen-thawed semen samples suggests that perhaps more stringent measures of hygenicity are required during processing. Alternatively the bacteria can even survive the deep-freezing and remain latent during storage at -196°C (Brown et al., 1974; Bielanski et al., 2003; Mazzilli et al., 2006).

In present study the frequency of appearance of bacterial genera demonstrated that fewer genera were present in samples treated with GTLS and their intensity was reduced compared to those of SP or NC (negative control; extender without antibiotics). These observations fit well with the data on TABC of this study where efficacy of GTLS was unequivocal in restricting microbial growth. Our findings regarding the frequency of occurrence of aerobic bacteria in bovine semen are in agreement with the earlier studies (Salisbury et al., 1978; Naidu et al., 1982; Aleem et al., 1990). The predominance of pseudomonas and *E. coli* in buffalo semen could perhaps be linked to lowered fertility usually observed in buffaloes (Aleem et al., 1990; Din et al., 1990). The 48 hours old cultures from buffalo semen of these organisms, when injected to mice through intra-peritoneal route, killed them within 24 hours (Aleem et al., 1990). Wierzbowski et al. (1980)

reported that pseudomonas and *E. coli* isolated from cattle bull semen had toxic effects on mouse embryo development. Incidentally, Parusov (1974) and Palli et al., (1975) reported that pseudomonas was resistant to SP.

The data on antibiotic sensitivity showed that majority of the bacterial isolates from buffalo bull semen samples showed more sensitivity towards gentamycin, tylosin, lincomycin and spectinomycin than streptomycin and penicillin. The resistance of pseudomonas to streptomycin and penicillin were also reported by Parusov (1974), Palli et al. (1975) and Aleem et al. (1990). Gentamycin was 100% effective against proteus, while penicillin exhibited no inhibitory effect. Similar findings were reported by Overgoor (1967) and Aleem et al. (1990). Penicillin showed maximum resistance against staphylococcus which is in agreement with the findings of Aleem et al. (1990). These findings were, however completely in disagreement with the results of Ahmed (1984) who reported 100% sensitivity of staphylococcus and proteus isolates to penicillin. It is reported that bacteria become resistant due to prolong use of same antibiotics for control of bacteriospermia (Alford, 1953; Parusov, 1974; Ahmad and Foote, 1985).

The pattern of sperm cell movement is sensitive to the chemical and physical properties of the medium in which they are suspended (Rasul et al., 2000). The motilities (visual, computer-assisted circular and linear) did not differ due to antibiotics in this study. These results are similar to those of Lorton et al. (1988a) who observed that GTLS had no adverse affect on post-thaw progressive motility of bovine spermatozoa tested at three commercial AI

(artificial insemination) centres. Percentage of post-thaw motile spermatozoa declined when concentration of gentamycin exceeded 500 µg/ml in milk and 1500 µg/ml in TCA (Tris-citric acid) extender (Ahmad and Foote, 1985). In an earlier study on buffalo, kanamycin (500 µg/ml) or ampicillin (250 µg/ml), tested individually did not affect the motility or liveability of spermatozoa in samples after 24 hours of storage (Hussain et al., 1990). Similarly, ampicillin (250 µg/ml) was found to be relatively better on motility and survival of buffalo spermatozoa than streptomycin and penicillin or gentamycin sulphate used in lower dose (Ali et al., 1994). Previously, kanamycin, erythromycin, streptomycin and terramycin were shown to be deleterious to mammalian spermatozoa, whereas gentamycin and lincomycin had no detrimental effects (Stallcup and McCartney, 1953; Back et al., 1975). Loss in motion characteristics of stallion spermatozoa due to increase in dose of antibiotics in extender has also been reported (Jasko et al., 1993). Mechanisms by which most of the antibiotics adversely affect sperm viability are through inhibition of protein synthesis, impairment of folic acid synthesis and intercalation with DNA (Gilman et al., 1985). Thus, GTLS in combination appears to be equally good as an alternate for cryopreservation of buffalo bull spermatozoa.

The measurement of velocity has been considered as an indirect indicator of mitochondrial function of a spermatozoon (Graham et al., 1984) and is associated with fertility (Budworth et al., 1988). Amplitude of lateral head displacement may indirectly reflects the efficiency of the flagellar beat pattern, appears to be a critical factor whenever the generation of shearing forces is

involved, as in sperm penetration of cervical mucus or their ability to penetrate the cumulus oophorus and the zona pellucida (Jeulin et al., 1986). Sperm motion characteristics, in this study, demonstrated that the mean velocities (straight-line, average path and curvilinear) and ALH (amplitude of lateral head displacement) did not differ due to antibiotics. These values are in general agreement with a previous study from our laboratory in which TCA buffering system containing conventional antibiotics (SP) was used for buffalo spermatozoa (Rasul et al., 2000). Indirectly, this suggests that GTLS preserved the mitochondrial and other cellular functions of the spermatozoa well. Amikacin sulphate and potassium penicillin most commonly sustained sperm motion characteristics in cooled equine semen (Varner, 1991). However, the speed of the sperm movement was affected adversely with increasing minocin concentration (Ahmad and Foote, 1984). Because the GTLS did not change the curvilinear velocity and ALH, motion characteristics associated with sperm capacitation and acrosome reaction remained the same.

The evaluation of plasma membrane integrity is of particular importance due to its involvement in metabolic exchanges with the surrounding medium. Furthermore, the process of capacitation, acrosome reaction and the oocyte penetration requires a biochemically active membrane (Jeyendran et al., 1984). Plasma membrane functional integrity of buffalo spermatozoa was evaluated using HOS (hypo-osmotic swelling) assay. The swelling ability of frozen-thawed spermatozoa in this experiment did not vary due to antibiotics,

suggesting that GTLS is not detrimental to plasma membrane integrity. Overall percentage was higher compared to those of Rasul et al., (2000) who used SP in freezing diluents. It is relevant to mention that gram-negative micro-organisms are reported to attach themselves to spermatozoal membranes (Bolton et al., 1986; Wolff et al., 1993). Bonadonna (1971) and Diemer et al. (1996) reported that binding of the bacteria resulted in micro-structural damage to sperm plasmalemma.

Morphological defects of spermatozoa, which have been associated with reduced fertility in bovine with AI, are abnormal heads, coiled tails and proximal droplets (Linford et al., 1976, Wood et al., 1986, Soderquist et al., 1991). According to Graham (1996), poor semen handling techniques or sub-optimal freezing conditions may induce irreversible morphological changes such as impulse of the sperm tail. In the present study, morphological abnormalities were less in case of GTLS and SP than NC.

The presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is being required at the proper time to facilitate fertilization (Thomas et al., 1997). The change in acrosomal cap is mainly due to sperm injury or aging (Saacke and Marshall, 1968). In the present study, the sperm cells possessing normal acrosome were higher in case of GTLS and SP than NC. In a similar pattern, acrosomal integrity was not significantly affected with GTLS compared to the combination of penicillin, dihydrostreptomycin and polymyxin B sulphate (Lorton et al., 1988a).

Findings of Gerard et al. (1995) are also in agreement with the present study, who reported that addition of GTLS to bovine semen extender, significantly improved intact acrosome as compared with the control without antibiotics. Damage to acrosome in NC may be due to bacterial growth. Presence of micro-organisms, especially the bacteria in the ejaculates can affect fertilization directly (Morrell, 2006), by inducing acrosome reaction (El-Mulla et al., 1996). Ahmad and Foote (1985) found that acrosomal integrity was increased with addition of gentamycin as compared to SP. Based on these observations, it appears, as if the events leading to cellular injury are of progressive in nature and changes first occur in the acrosomal morphology.

The *in vivo* fertility results of present study are in line with previous studies which have shown that GTLS as a component of various semen extenders had no negative influence on pregnancy or non-return (NR) rates in bovine when compared with other antibiotics used alone or in combinations. Stoyanov (1987) reported that gentamycin is best antibiotic for bull semen extender as compared to penicillin or streptomycin in terms of higher fertility rates. Whereas, Lorton et al (1988b) and Sullivan et al. (1988) reported no significant effect on seminal quality as measured by field fertility using GTLS or dihydrostreptomycin, penicillin and polymyxin B sulphate with or without linco-spectin in heated whole-milk or egg yolk-sodium citrate extenders. More recently, Bousseau et al. (1998) reported a similar trend for *in vitro* and *in vivo* fertility tests conducted with GTLS-based Biociphos plus®extender and Laiciphos® extender containing SP plus linco-spectin. The significant

difference in fertility rate in present study can be attributed to the use of only two antibiotics in combination (SP) vs. GTLS.

Kupferschmied et al. (1991a) found no difference in fertility rate in cows on using SP or GTL with or without spectinomycin in Tris-diluents prior to deep freezing. Kupferschmied et al. (1991b) also reported no significant difference in NR rates in cows inseminated with frozen-thawed semen containing either SP or GTLS. Better efficacy of GTLS in present study could be due to difference in method of addition of antibiotics in semen diluent. Also in current study, the lowered fertility rates in buffaloes observed with SP 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 SP (Aleem et al., 1990). Results of Shin et al. (1988) have indicated that GTLS has a broader spectrum of microbial control in frozen bovine semen than SP with or without polymyxin B. Thus, 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 seminal quality/fertility (Lorton et al., 1988b). Influence of GTLS has also been compared after *in vitro* fertilisation and culture of bovine embryos, with higher cleavage rate for GTLS-containing Biladyl® (TCA) extender (Lonergan et al., 1994). In contrast to our findings, Kommisrud et al. (1996) reported significantly higher NR rates with SP-containing skim milk-egg yolk extender as compared to GTLS-based Biladyl® extender in cattle. This variation may possible be attributed to the difference

in semen extenders and dilution methods. Other then semen quality, the fertility rates are affected by a number of other factors including female reproductive status and genetic, management and nutrition (Younis et al., 1999; Graham and Moce, 2005). Also this variation might be due to technical know how, season and geographical area (Rodriguez-Martinez, 2003).

In conclusion, bacterial and seminal quality measured by standard laboratory tests and field fertility trial indicates that GTLS combination of antibiotics is more suitable in semen extender for cryopreservation of buffalo bull spermatozoa.

The GTLS combination of antibiotics can be incorporated into a freezing extender/protocol without compromising the post-thaw quality and fertility of buffalo bull spermatozoa. Hence GTLS is recommended for routine use in the semen production units for improving the AI efficiency in buffalo with cryopreserved spermatozoa.

For future studies polymerase chain reaction (PCR) based identification of bacterial species in buffalo bull semen is recommended. Epidemiological study is also recommended for occurrence of bacteriospermia in buffalo. Further that testing of wider range of new antibiotics is recommended for cryodiluents of buffalo bull spermatozoa.

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