Dissertation Ref. No. _____

QUALITY OF SEMEN AND ITS IMPLICATION ON FERTILITY OF BREEDING BULLS: A MULTIPARAMETRIC ANALYSIS USING COMPUTER ASSISTED SEMEN ANALYZER AND FUNCTIONAL TESTS OF SEMEN PRODUCED AT LIVESTOCK DEVELOPMENT INSTITUTE

PhD Dissertation

By

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Addis Ababa University, College of Veterinary Medicine and Agriculture
Department of Clinical Studies
PhD Program in Veterinary Obstetrics and Gynecology

April, 2022
Bisheftu, Ethiopia

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A Dissertation Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial fulfillment of the Requirements for Doctor of Philosophy in Veterinary Obstetrics and Gynecology

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As members of the Examining Board of the final PhD open defense, we certify that we have read and evaluated the Dissertation prepared by Kefelegn Seyoum, titled: "Quality of semen and its implication on fertility of breeding bulls: a multiparametric analysis using computer assisted semen analyzer and functional tests of semen produced at Livestock Development Institute", and recommend that it be accepted as fulfilling the dissertation requirement for the degree of Philosophy in Veterinary Obstetrics and Gynecology.

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Addis Ababa University (2022)

ABSTRACT

Various factors are believed to influence the quality of semen in breeding bulls. Among them breed, bull selection and management, diseases and evaluation methods are believed to be the most important one. Poor semen evaluation, such as used in the conventional methods that do not address aspects of the complex spermatozoon structure and subsequent fertilization process implies the production of semen with low conception rate compromising success of artificial insemination. Reports confirm that the Ethiopian Livestock Development Institute (LDI) uses the conventional method and produces semen characterized by low conception rate. A multiparametric semen evaluation using advanced technology and functional tests that sets a higher standard for approval would improve the quality of semen. Accordingly, a computer based integrated semen analysis system, and acrosomal and plasma membrane integrity tests were applied on semen collected from 14 breeding bulls (Boran = 4, Boran Holstein Frisian Crosses = 4 and Holstein Frisian = 6). Bulls were additionally investigated for incipient general and reproductive diseases. After the initial conventional assessment, semen was evaluated using advanced multiparametric method at fresh, chilled and frozen stages for various semen quality parameters. There were significant ($P < 0.05$) breed differences between Boran and Holstein Frisian breeds in scrotal circumference (33.20 Vs 38.40cm), testes volume (254.40 Vs 367.50cm^3), testes weight (264.10 Vs 381.40g), semen volume (9.81 Vs 11.39ml), pH (6.31 Vs 6.58), spermatozoa head abnormality (5.02 Vs 3.42%), Hypo osmotic swelling reactivity (39.55 and 49.46%) and acrosome intact live spermatozoa*

(43.29 and 49.67%), respectively. Total sperm morphological abnormality (18.10%) were significantly (P < 0.05) higher in the Crosses. Motility was significantly higher (82.5%, P < 0.05) in fresh Boran semen while the same parameter was higher in chilled (70.2%) and frozen (42.9%) semen from HF bulls. All kinematic parameters (VCL, VAP, VSL, ALH, BCF, LIN, STR, and WOB) were significantly different (P<0.05) at all stages of semen production among the three breeds, being lower for Cross breed bulls. The ISAS method and the functional tests applied in this study invalidated some of the values of sperm parameters from the conventional method. Moreover, results of HOST, acrosome integrity and sperm morphological defects were associated with one or more of sperm motion characteristics which were not revealed if only conventional method was used. With minimum cut-off values (total motility=40% and progressive motility=15%) in frozen semen, nearly 56% of semen produced at LDI failed to fulfill the minimum approval requirements by ISAS. This was very apparent in semen of Cross bulls for both parameters (total motility 33.1 Vs 12.9% progressive motility) and to that of total motility (33.9%) in Boran semen. All bulls investigated were found to have no general and/or reproductive health problems that influenced the quality of semen. It is therefore plausible to assume that poor semen quality resulting from absence of a more rigorous evaluation method could be one of the factors contributing to the low conception rate at the field level. The significance of introducing advanced methods of semen evaluation is incontrovertible; however, this finding has to be complemented with more field studies and investigation of risk factors in the female individual to get a complete picture. As LDI is bearing a huge national responsibility in advancing the cross breeding program based on AI, it becomes invariably mandatory to start using state of the art methods of breeding soundness examination and semen evaluation.

***Key words**: Acrosomal integrity, Boran, conventional method, Crosses, Holstein-Frisian; HOST, ISAS, semen quality, sperm kinematics*

STATEMENT OF AUTHOR

First, I declare that this dissertation is my bonafide work and that all sources of material used for this dissertation have been duly acknowledged. This dissertation has been submitted in partial fulfillment of the requirements for a PhD degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this dissertation is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Date of Submission: June/2022

ACKNOWLEDGEMENTS

First of all I would like to give my special thanks to my advisor Prof. Alemayehu Lemma for his guidance, constructive comments and careful correction of the dissertation.

I would like to thank Addis Ababa University thematic project, Collage of Veterinary Medicine and Agriculture, and the Livestock Development Institute, in particular the general and deputy general directors of the institute for their willingness to do the research on semen producing bulls.

I would like also to extend my acknowledgement to the lab personals of the institute; Dr. Bula Agegnehu, Mr. Million Adimasu, Mr. Tesfaye Yigezu, W/rt Workinesh Abera, W/rt Etsegenet Mekonnen, W/r Meaza Debash, Mr. Gossa Dibaba, Mr. Jiregna Ayele, Mr Zemedkun Teketel and W/rt Wineshete Teklie for their respectful collaboration in the actual laboratory work. I would like also to deserve special thanks for the contribution and assistance of all bull stud workers.

I extend my sincere thanks to Dr. Tilaye Demessie whom stand with me as elder brother in all challenging situations of the study period.

I also want to thank others who directly and indirectly assisted me during the entire program of the work.

DEDICATION

This dissertation is dedicated to my beloved Dad Seyoum Abebe and Mam Amsale Temetemie, whom I lost on 17 April 2009 and on 10 February 2013. May God rest their soul in peace!

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LIST OF ABBREVIATIONS

AI	-	Artificial Insemination
AID	-	Acrosome Intact Dead
AIL	-	Acrosome Intact Live
ALD	-	Acrosome Lost Dead
ALH	-	Amplitude of Lateral Head displacement
ALL	-	Acrosome Lost Live
BCF	-	Beat Cross Frequency
BVD	-	Bovine Viral Diarrhea
CASA	-	Computer Assisted Semen Analyzer
CSA	-	Central Statistical Agency
HOST	-	Hypo Osmotic Swelling Test
IBR	-	Infectious Bovine Rhino trachitis
ISAS	-	Integrated Semen Analysis System
LDI	-	Livestock Development Institute
LH	-	Luteinizing Hormone
LIN	-	Linearity
PNA	-	Arachis hypogaea agglutinin
PPD-A	-	Purified protein derivative of Avian
PPD-B	-	Purified Protein Derivatives of Bovine
PSA	-	Pivum Sativum Agglutinin
PUFA	-	Poly Unsaturated Fatty Acids
STR	-	Straightness
TB	-	Tuberculosis
VAP	-	Average Path Velocity
VCL	-	Curvilinear Velocity
VSL	-	Straight Line Velocity
WOB	-	Wobble

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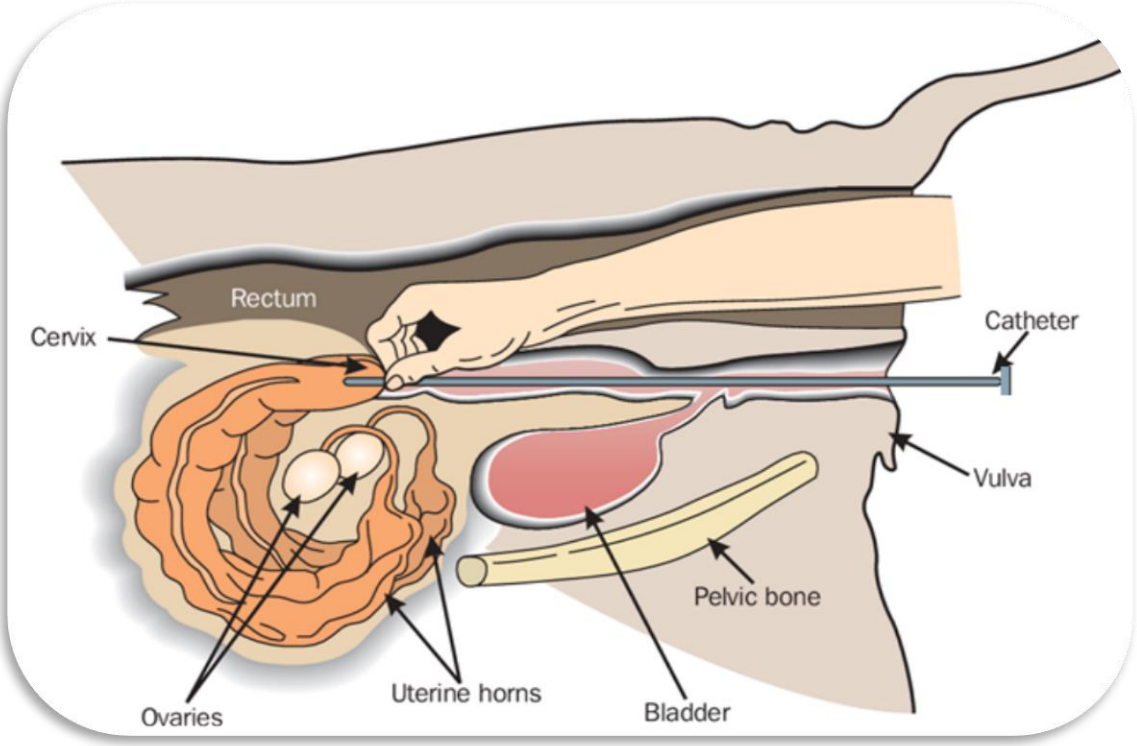
Chapter I



INTRODUCTION

Ethiopia has one of the highest (70.29 Million) cattle population reared under diverse agro climates. However, of this population 97.4% are unimproved indigenous breeds and the remaining 2.6% breeds are considered to be hybrids (2.29%) and Exotics (0.31%) (CSA, 2021). Concomitant with this huge potential and its first rank in Africa the contribution to the economy is limited and not to the expectation (Kassahun, 2008). A number of complex and inter-related factors like poor genetic potential, inadequate feed, widespread diseases and inadequate livestock development services with respect to credit, extension, marketing, and infrastructure have contributed to this (Negassa and Jabbar, 2007; Rich *et al*., 2011).

Considering reproduction, a bull has a greater impact on the herd productivity than a single female especially when it is used for artificial insemination (AI). Each bull can serve thousands of females per year. AI has a huge role for the genetic improvement programs, when it is practiced through a well-established methods of identifying the breeding males with the highest fertility potential/genetic merit (Leboeuf *et al.*, 2000). At present, frozen-thawed semen is extensively used for AI in animal breeding throughout the world (Ball and Peters, 2004). Because of its relative simplicity for application and its contribution in speeding up genetic improvement, it is also the first and most commonly used reproductive technology in Ethiopia.

Many factors are known to affect the efficiency of AI in field condition. However, the proper selection of fertile bulls and the ability to produce the best quality semen determine the success of AI (Holt *et al*., 2007). Variations in breed, age, nutrition, season (heat stress), and sexual behaviors that influence the fertility of the bull have been frequently associated with quality of semen (Javed *et al.,* 2000; Freneau *et al.,* 2010; Dhurvey *et al.,* 2012; Gopinathan *et al.,* 2018). As it was described by Zubair *et al*. (2013), though a wide variety of diagnostic tools are used to investigate the quality of semen in bulls, it is still difficult to determine to what extent sub-fertile semen contributes to failure of conception. As it was also stated by Tanga *et al.* (2021), no single test can precisely determine the fertility potential of semen. Hence, diversifying the evaluation

approaches to semen quality is one of the options for an accurate estimation of fertility potential of the ejaculate. Conventionally; percent of moving spermatozoa by visual approximation under phase contrast microscope has been used to quantify motility. Whereas; analysis of semen based on different parameters/assays using advanced methods such as computer assisted semen analyzer (CASA) and functional tests can provide better information about the semen quality and its fertility potential (Madeja *et al.*, 2003).

CASA evaluates semen samples objectively and provides an opportunity to assess sperm kinetics more precisely, rapidly and accurately. As CASA yields repeatable and highly reliable results on motility percentage, motion characteristics and kinematics of individual sperm cell; it is more dependable in predicting the fertility potential of a bull (WHO, 2010; Agnieszka *et al.*, 2012; Lu *et al.*, 2013).

In Ethiopia, the former National Artificial Insemination Center (NAIC) later named National Animal Genetic Improvement Institute (NAGII); and more recently renamed as Livestock Development Institute (LDI) was established in 1981 to coordinate the overall bovine AI operation in Ethiopia. The institute is responsible for importing bulls and in-calf heifers that are used mainly for the dairy development. To complement its role, LDI also selects indigenous breed bulls for semen production based on their genetic merit. However these huge responsibilities of the institute have rendered its service inefficient; as studies showed, manifested by the very low conception rate of cows and heifers served by the semen produced from the institute. Pregnancy rate reports from different regions of the country ranges from 7.1% in Tigray to 42.9% in SNNP regions and 27% at a national level (Gebre Medhin *et al.*, 2007; Jemal, 2012). The post-thaw progressive motility evaluated at field level has been as low as 20% which is below the recommended 40% set by the institute itself (Gebre Medhin *et al.* (2007). Moreover, data on the reproductive and fertility potential of zebu and crossbred bulls are insufficient. The current practice at LDI is also just using conventional method of semen evaluation which is not adequate to predict the fertility potential of the bulls. So far several studies (Sori, 2004; Sinishaw, 2005; Gebre Medhin *et al.*, 2007; Demeke, 2010; Engidawork, 2018) also undertook the

same subjective assessment to determine quality of semen in an attempt to predict the fertility potential. None have used advanced technologies or functional tests at a time to objectively evaluate the semen quality to predict fertility potential. Recent use of Boran semen produced at LDI for *in vivo* embryo production resulted in the production of very high proportion of unfertilized ova (UFO) compared to imported HF semen (Degefa, 2016). There have been anecdotal data indicating a dismally low (<30%) pregnancy rate from unimproved Boran breed in Ethiopia. On the other hand, there has been a huge demand by the Ethiopian government for Boran semen to propagate the cross breeding program. However, semen quality seems to have hampered its wider use both for AI and Embryo Transfer (ET). In the present study, it is hypothesized that employing a stringent breeding soundness examination and advanced techniques of multiparametric semen evaluation invariably improve semen quality and hence fertility potential of bulls. Grounded on this hypothesis; the main goal of this research was to evaluate how application of a more stringent methods of semen quality assessment can influence fertility of breeding bulls The Specific objectives were:

- To undertake stringent breeding soundness examination for the three semen producing breeds of bulls used for semen production at LDI to evaluate its implication on fertility
- To evaluate the semen quality of Boran, Crosses and HF breeds using a computer assisted semen analysis system and to see the advantages of introducing advanced multiparametric semen evaluation techniques on improving quality of semen in particular from indigenous and their cross breed bulls
- To assess the structural and functional integrity of the acrosome and plasma membrane, and thus to study the role of these tests in improving the quality and fertility of semen from those breeds
- To study role of general and reproductive health problems caused by IBR, BVD, TB and Brucellosis on the quality of semen produced at LDI.

Chapter II

LITERATURE REVIEW

2.1. Breeding Soundness Evaluation (BSE)

The bull is half of the herd; any abnormality in the breeding bull will cause a catastrophic loss to the breeding program. Therefore, a proper breeding soundness evaluation must be in place. Bull breeding soundness evaluation reduces the risk of using sub fertile/infertile bulls in the semen production center; hence it decreases unnecessary costs and improves strategic bull usage (Lone *et al*., 2017). Essential components of BSE include undertaking complete general and reproductive physical examination, conducting libido testing and accurate evaluations of semen quality. As it was described by the authors, bulls passing a BSE and/or related semen quality tests, have a 6% or higher fertility as compared to unevaluated bulls. Therefore, in order to categorize the bulls as satisfactory potential breeders, they must pass the physical examination and attain the minimal thresholds for libido testing and semen quality evaluations.

2.1.1. Physical examination of bull

A complete general and reproductive physical examination of the bull includes examination of body parts such as the head, eyes, legs, feet, hocks, prepuce, scrotal or testicular abnormalities/injuries and the overall physical appearance. Moreover, detection for seminal vesiculitis, or prostate abscess formation on trans-rectal examination and examinations of penile and prepucial tumors, lacerations, warts, scars and frenulum which may interfere with coitus are the essential considerations taken into account while the general and reproductive physical examination of bull is conducted (Lone *et al.*, 2017).

The bull reproductive system consists of primary sex organs (testes), tubular secondary sex organs (efferent ducts, epididymes, vasa differentia, urethra and penis) those transport semen from the testes to the exterior and accessory sex glands (seminal vesicles, prostate and bulbourethral glands). During the examination of genitalia, all parts accessible externally and internally (like sigmoid flexure and accessory sex glands) should be palpated for their size, consistency, resilience, evenness and abnormalities (Jordan, 2021).

The testes consist of a number of elongated *seminiferous tubules* which are surrounded by connective tissues containing large polyhedral cells (*cells of Leydig)* with large nuclei and granular cytoplasm (Jordan, 2021). Small tubular vessels (vasa efferentia) from each testis open into long convoluted epididymis. The epididymis is a duct system with muscular wall that is important for propulsion of sperm cells through it. The epididymis gathers the sperm cells produced in the testis and serves a site for storage and maturation of spermatozoa. Epididymis is divided into three segments (head, body and tail) those can be palpated during the physical examination of testes. Head of the epididymis is a wide flattened structure positioned at the top towards the front of the testis. The head then narrows into body of the epididymis:- a flattened tube that travels down the inner side of the testis to the bottom where it forms the tail. Tail of the epididymis is an enlarged area that acts as a a storage and maturation for spermatozoa. At its end, it makes a hairpin smaller diameter tube called the deferent duct (*vasa deferentia)* that travels back up along the inside surface of the testis and continues up to the urethra just after the bladder opening near the site where the seminal vesicles join with the urethra. The walls of *vasa deferentia* are again muscular and undergo rhythmic contractions for transport of sperm cells (Jordan, 2021).

Seminal vesicles are multilobate glands that are usually 8 to 15 cm long and 2 to 4 cm wide and lie cranially to the prostate gland. However, size of seminal vesicles is related to age of the bull and seminal vesiculitis (Bromfield *et al.,* 2018). Prostate is a circumferential gland located in between bulbourethral glands and seminal vesicles. Both the seminal vesicles and prostate gland are easily palpated trans-rectally. The bulbourethral glands lie dorsally to the penis and are located close to the ischial arch. According to the authors, the secretion of accessory glands is essential for initiation of sperm cell motility and also provides a supportive and nutrition rich environment for the spermatozoa.

2.1.2. Libido testing

Libido is considered to be highly heritable in cattle and the high test scores of libido have association with good reproductive performance (Quirino *et al.*, 2004). For this reason assessment of libido and serving ability is widely used for predicting fertility potential of bulls. However, as it was described by Chenowith (2012), though rankings are much more consistent; individual bull's libido score is not highly repeatable. Moreover, factors like age, sexual experience and social dominance also affect libido and decrease the test reliability. In consequence, some earlier researchers (Hopkins and Spitzer, 1997) encouraged clinical examinations rather than libido testing.

In most of the time lack of libido is seen either for young or advanced aged bulls. The conditions under which a young bull has been reared also affect its behavior. Bull calves reared in groups, continuously exhibit mounting behavior as puberty approaches and usually learn to copulate quite quickly. Whereas, those reared in isolation, such mounting behavior does not occur and can seem to take time to learn, especially in AI stations; where steers are the sole objects available for the young bull to mount (Chenowith, 2012).

Most lesions affecting locomotion also impair the ability and willingness to copulate (Lone *et al.*, 2017). Lesions of the back and hind legs are the most important of such incapacities. Moreover, overgrown hooves of a bull adversely affect the distribution of the bull weight while mounting, and enforce the bull to become unwilling to mount; or even, if it mounts, it will not remain mounted for long enough successful copulation. In most aged bulls, lesions of the joints of the hind limbs are also important locomotor causes of impaired libido (Lone *et al.*, 2017).

2.1.3. Semen quality

The most representative method to evaluate semen fertilizing capability is through the results obtained by *in vivo* fertility. However, this is very difficult and is time taking to practice. For this reason, most semen producing laboratories have been used conventional

semen evaluation methods through the provision of appropriate preparation and precautions to evaluate and predict semen quality (volume, color, concentration, motility viability and morphology) and fertility potential. However, due to the complex structure of spermatozoa and fertilization process, usefulness of these conventional methods to predict fertility is limited (Chenoweth *et al.,* 2010; Beggs, 2013).

Semen is composed of spermatozoa and a liquid part called seminal plasma (Barszcz, 2012). However, its composition depends on the level of development of accessory reproductive glands, the share of secretions from reproductive organs/ glands and the volume of spermatozoa in total. According to the author, in the bull semen, the secretions were from: seminal vesicle (50%), bulbourethral glands (25%), epididymis (7%), prostate gland (5%), and the remaining 13 - 14% are expected to be spermatozoa.

2.2. Factors Affecting Bovine Semen Quality

Semen quality of a bull is affected by different factors such as age, breed, nutrition, temperature and season at which semen is collected, diseases in particular of testis, epididymis and accessory glands, scrotal circumference and other possible factors like trace elements, ejaculate frequency, collection technique, handling procedure during and after collection, analytical techniques and variation among technicians and pharmacologic agents are some of the factors those have impact on semen quality (Javed *et al.,* 2000; Freneau *et al.,* 2010; Health *et al.,* 2011; Dhurvey *et al.,* 2012; Gopinathan *et al.,* 2018).

2.2.1. Age

Several researches agreed the improvement of ejaculate volume, sperm concentration and motility with the advances in bull age ((Javed *et al.,* 2000; Vilakazi and Webb, 2004 Health *et al.,* 2011; Lemma and Shemsu, 2015; Andre *et al.,* 2017; Sitali et al., 2017; Ahmed *et al*., 2018; Gopinathan *et al.,* 2018; Seyoum *et al.,* 2021). Most these authors agreed as bulls of 2-5 years of age produce a relatively better semen quality than their cotemporary of less than 2 and older than 5 years of age. An earlier study conducted by

Salisbury *et al.* (1978) also notified the effect of age on semen quality; and stated as production and quality of semen start to increase beyond the first year after puberty. These researchers found a correlation of 0.51 between sperm production and age at first pubertal life in Holstein bulls.

Spermatozoa morphology is one of the semen quality traits and often used as important criteria in the evaluation of semen quality in domestic animals which is also affected by age of bull (Wolfenson *et al.,* 2000; Vilakazi and Webb, 2004; Freneau *et al.,* 2010; Andre *et al.,* 2017). An increase in abnormal sperm cell count with advanced aged bulls which has been reported by Wolfenson *et al.* (2000); that stated bulls at the age of 6 and 7 years produce fewer normal structural sperm than their younger counterparts. Older bulls have higher incidence of sperm abnormalities than young bulls (1.5-5 years). These authors also noted as younger bulls (less than 13 months) had higher incidence of sperm head abnormalities and proximal cytoplasmic droplets than bulls of 1.5-5 years of age. Likewise, Vilakazi and Webb (2004) also reported that bulls aged 3 to 4 years were found to produce sperm of better morphology than bulls of 6 years of age and older.

2.2.2. Breed

Influence of breed on semen quality had been investigated by different researchers (Brito *et al.,* 2002; Andre *et al.,* 2017; Centre, 2017; Seyoum *et al.,* 2021). According to Brito *et al.* (2002), *Bos indicus* bulls have a significant higher sperm concentration but with higher sperm morphologic defects than *Bos Taurus* bulls. Libido and mating ability of bull have also been influenced by genetic factor. Lemma and Shemsu (2015) in their research finding have been reported the semen characteristics difference among local breeds. Likewise, Seyoum *et al.* (2021) also investigated the significant influence of breed on semen ejaculate volume, mass activity, individual motility, concentration and production doses per ejaculate.

2.2.3. Scrotal circumference and testicular measurements

The scrotal circumference measurement is carried out during general and reproductive examination of bull and it is one of the parameters to be considered for breeding soundness evaluation. Hence, as it is relatively accurate and easy to take its measurement and is highly correlated with testicular volume and sperm output (Lone *et al.*, 2017); it can be used for the selection of bull at young age. Depending on the individual's preference, facilities and the bull's temperament, scrotal circumference can be measured from the side or rear of the bull. Procedurally, as it was described in Lemma and Shemsu (2015), scrotal circumference measurement can be conducted by pushing the testes firmly into the bottom of the scrotum while placing the thumb and fingers laterally on the side of the neck of the scrotum to make them completely within the lowest point in the scrotum and lying side by side with no evidence of wrinkling of the scrotum. The testes are then held firmly in the scrotum with left hand and measurement of scrotal circumference is taken with right hand by taking care for the thumb of the hand holding the neck of the scrotum not to cause any pressure on the middle of the scrotum. The scrotal tape is then looped around the testes and can be drawn firmly in contact with the entire circumference to cause moderate indentation of the scrotum at the level judged to have the largest circumference. According to Beggs (2013), the normal spermiogram of semen producing bulls was most likely associated with scrotal circumference measurements between 31 and 44 cm. Likewise, as to the description of Chenoweth (2012), if scrotal circumferences in a given bull population follow a normal distribution; then the bulls at the bottom of the curve are linked with decreased semen quality or fertility. Furthermore testicular length, width, thickness, and volume have also their own additional importance in predicting the fertility potential of the bull and technically these parameters can be measured as follows described by (Bailey *et al.*, 1998).

Testicular length is measured by placing the fixed arm of the caliper at the proximal end and the sliding arm at the distal end of the testis being taking care to exclude the epididymis. Likewise, testicular width of each testis is measured by sliding the other testis up in the scrotum and placing one arm of the caliper at the medial aspect and the other at

the lateral at the point of maximum width. Similarly, testicular thickness is measured by placing the fixed arm of the caliper at the anterior aspect and the sliding arm at the posterior aspect of each testis at the point of maximum thickness. Bailey *et al.* (1998) also developed a technical formula to calculate the volume of testes on live bulls and stated as;

$$V = \frac{4(\pi abc)}{3};$$ Where: a = thickness/2, b = width/2 and c = length/2.

2.2.4. Nutrition

Nutrition given at pre and post-pubertal stages of development has effect on bovine semen quality (Dance *et al.,* 2016; Kenny *et al.,* 2018). According to Dance *et al.* (2016), improving the nutritional requirement up to the age of 8 months increases the number of spermatozoa to be harvested post-pubertly in bulls. Kenny *et al*. (2018) also stated the effect of enhancing plane of nutrition of bull calves during the first 6 months of life that can increase gonadotropin secretion and testicular development, which in turn result in earlier onset of puberty.

Post-pubertal nutrition also plays a role in maintenance of normal semen production (Kenny *et al*., 2018). High plane of nutrition (cereal-based diet) that has association with the increment in scrotal fatness and temperature can negatively impact progressive motility and sperm morphology in bulls (Coulter *et al.,* 1997). Moreover, rapid introduction to concentrate-based diets, which are rich in readily rumen fermentable carbohydrate results in a reduction in ruminal pH that can result sub-acute or even acute ruminal acidosis (SARA); this in turn results reduction in percentage of morphologically normal sperm with large increment for proximal droplets, knobbed acrosome and vacuoles (Kenny *et al.,* 2018). According to the authors, feeding cattle with high concentrate-based diets also leads to an increased incidence of laminitis which in turn adversely affect the distribution of the bull weight while mounting, and enforce the bull to become unwilling to mount and even if it mounts, enforces not remain mounted for long enough successful copulation. Thus feeding cattle with high concentrate-based diets has influence on semen quality. Moreover, inadequate supply of sulphur containing amino

acids, methionine and cysteine may increase incidence of lameness as a result of the formation of soft hooves. This was also supported by Persson *et al*. (2007) for bulls culled because of infertility in Sweden; that reported 67% of the 34 infertile bulls had moderate or severe lesions associated with osteoarthritis compared with non-lamed 11 contemporary bulls.

According to Kenny *et al*. (2018) feed deficiency has also adverse effect on physiological mechanism of testes, epididymis, and seminal glands and results semen characteristics in particular of semen volume and total spermatozoa to be affected in bulls.

2.2.5. Effect of season and temperature

Several studies (Javed *et al.,* 2000; Dombo, 2002; Vilakazi and Webb, 2004; Brito, 2010; Health *et al.,* 2011; Mishra *et al.,* 2013; Andre *et al.,* 2017; Gopinathan *et al.*, 2018; Prem Kumar *et al.,* 2020) showed the effect of season on semen quality. Season includes factors (like temperature, humidity and feed quality) that have influence on semen quality. Differences in the quantity and/or composition of feed, environmental temperature and humidity affect semen output. Hirwa *et al.* (2017) also support the variation of semen output, quality and mating behaviour of bulls from season to season depending on availability and quality of feed and climatic conditions in the tropics.

Scrotal temperature plays a major role in determining production and quality of semen (Nilani, 2016; Centre, 2017; Hájek, 2017). Deep body temperature is too warm for proper sperm production. That is why the testes are located outside the body core. As the environmental temperature changes, the testes are either raised or lowered in the scrotum to maintain proper temperature for sperm production. However, under high ambient temperature, body thermoregulatory mechanism is unable to increase body heat loss; hence internal temperature increases above the physiological limit (Centre, 2017).

Though individual bull response to thermal stress is different; semen quality tends to be lower during summer months in the area where there is marked seasonal variation in the

environmental temperature (Vilakazi and Webb, 2004). According to the authors the increased abnormal sperm cells were recorded with extreme environmental temperature. Contrary to this, Jimenez-Severiano *et al.* (2003) and Hirwa *et al.* (2017) investigated the influence of season on the secretion of LH and concentration of testosterone which in turn govern the sperm production per ejaculate in bulls; and according to the authors the highest luteinizing hormone (LH) and testosterone concentration was recorded in spring and summer seasons than the cold winter period in young bulls.

According to Mridula *et al.* (2018), the optimal environmental temperature for spermatic production is estimated to range between 15 and 20 °C. Hence it is important to consider not only the temperature registered in the day of semen collection, but also the temperature registered during the entire spermatogenesis before collection that has effect on semen quality.

2.2.6. Disease risks with bovine semen

The two major importance of AI are to achieve continuous genetic improvement and prevent venereal diseases. Moreover, in comparison with natural service, few selected males are also needed to artificially inseminate females. However, unless proper and strict disease testing for such selected males have not been taken in to considerations, the risks associated with AI for its potential to spread diseases and genetic defects are unquestionable.

Disease causing agents may be acquired from infected animals to others through different routes like venereal, respiratory, digestive, etc. As a result infected bull fertility may be affected temporally or permanently depending on the type of infectious agent and the lesions produced on reproductive tract/organs. Hence infection could also be limited to a single organ or it might spread extensively to organs/glands.

According to Joya *et al.* (2011), diseases whose presence and/ or transmission associated with semen have been grouped into two categories: those whose presence and

transmission through semen have already been demonstrated (Infectious Bovine Rhinotracheitis, Bovine Virus Diarrhoea, Foot and mouth disease, Vesicular Stomatitis, Papillomatosis, Leptospirosis, Tuberculosis, Paratuberculosis, *Mycoplasma*, Anaplasmosis, Brucellosis, Campylobacteriosis and Trichomoniasis) and those whose presence in semen have been demonstrated but not their transmission (like Babesiosis, Trypanosomiasis).

Brucellosis: According to Givens and Marley (2008), infection of bull with brucellosis leads to reduced libido, lower semen quality and infertility. Contaminated semen could transmit infection when AI is used. In supporting this idea, the study of Campero *et al.* (1990) for experimentally inoculated bulls also verified the presence of *B. abortus* strain 19 in semen which was accompanied by a specific antibody response. However, according to the authors; there was no overt increase in seminal immunoglobulin (Ig) concentration and no definite conclusions could be made concerning the protective role of seminal antibodies in limiting spread of infection at service.

Isolation/demonstration of the organism in tissues or fluids and serological/agglutination tests on blood, milk or seminal plasma are the laboratory tests used to investigate of the disease incidence. A specific and sensitive PCR assay has also been developed to diagnose it. Some authorities in *Brucella-face* countries consider as serum agglutination test is sufficiently sensitive for health certification of Al bulls. However, semen or seminal plasma testing can be carried out for presence of the organism instead of serum agglutination test (Campero *et al.*, 1990).

Bovine Tuberculosis: Bovine Tuberculosis is commonly a chronic debilitating infectious disease caused by *M. bovis*, and is usually characterised by formation of nodular granulomas known as tubercles. As to the description of Allen *et al.* (2010), the bacteria associated with the disease may lie dormant in an infected animal for years without causing clinical signs or progressive disease symptoms and can reactivate during periods of stress or in older animals. Any body tissue can be affected, but lesions are most frequently observed in the lymph nodes (particularly of the head and thorax), lungs,

intestines, liver, spleen, pleura, and peritoneum (Allen *et al.*, 2010). According to the authors, though inhalation of micro-droplets in aerosols from infected animals and ingestion of contaminated food and water are the most common ways for the spread of the disease; shedding of the bacteria in feces, milk, and sometimes in vaginal secretions, semen or urine is also expected.

Infectious Bovine Rhinotracheitis (IBR): This is the most important viral respiratory disease caused by bovine herpesvirus type-1 (BHV-1). Because of the viral latency, infected animals become carriers for life and stress factors can cause frequent viral reactivation. As it was described by Fauquet *et al*. (2004), when BHV-1 affects the genital tract of cattle; it causes infectious pustular vulvovaginitis or infectious pustular balanopostitis. As it was also described by Takiuchi *et al*. (2005), it may also cause conjunctivitis, reproductive disorders and neonatal mortality. Moreover, infected bulls may show pyrexia, uni/bilateral orchitis and azoospermia. Different authors, (Joya *et al.*, 2011) recognized the transmission of this virus through semen or embryos. However, as to the description of Eaglesome and García (1997) the virus cannot eliminate from seropositive bulls even under low level of management.

Bovine viral diarrhea: This viral disease of cattle is caused by bovine viral diarrhea virus (Ridpath, 2010), and as it was described by Kirkland *et al*. (1997), BVD virus can replicate itself in the prostate, seminal vesicles and epididymis. Borel *et al*. (2007) also strengthen this idea, by detecting the virus antigen in epithelial cells from the epididymis, accessory glands, urethra, Sertoli cells and spermatogonia. Similarly researchers Givens *et al*., 2003) approved the presence of the virus in the testes and semen. The authors showed as the virus stays in the testes for up to seven months following initial infection. Likewise other researchers; Voges *et al*. (1998) also presented the elimination of the virus from semen during eleven months period in the presence of active antibodies. That is why Persistently-infected animals are those representing the highest risk of BVD transmission through semen since viral elimination from semen is much higher than from acute infections (Bielanski, 2007). The effect of the virus on semen quality has been established

using experimentally infected bulls, where semen showed low concentration and motility with increased frequency of primary spermatic abnormalities.

2.2.7. Other factors

Factors like deficiency of trace elements, frequency of service/ejaculation, collection techniques, semen handling procedures during and after collection, freezing techniques, analytical techniques, subjective variation among technicians, pharmacologic agents (like extenders & preservatives) and physiologic variations among bulls have been recognized to influence semen quality (Blezinger, 1999).

Semen is composed of cellular component (spermatozoa) and fluid medium (seminal plasma); and its seminal plasma again contains a complex range of organic (like proteins, fructose, flavin) and inorganic (Zinc, Copper, iron, selenium, calcium, magnesium, Manganese) constituents (Barszcz, 2012). As dealt by different researchers (Yuyan *et al.*, 2008; Sordillo and Aitken, 2009), oxidative stress is a major cause of infertility in mammals, but trace elements such as copper, iron, selenium and zinc are components of enzymes which have antioxidant activity. Thus such elements play a vital role in the reduction of fertility problems. For instance, Zinc can stabilize the DNA of spermatozoa; while its deficiency can bring lowered fertility because of the increased sperm fragility and adverse effect of spermatogenesis (Colagar *et al.,* 2009). Likewise, deficiency of Copper associates with production of smaller volumes of ejaculate, lower sperm concentration, lower motility and morphological changes. Iron is also an essential component of some proteins that are present in Leydig and Sertoli cells those are vital for hormone production and spermatogenesis (Yuyan *et al.,* 2008). As it was described by Xu *et al.* (2003), selenium is related to the sperm maturation in the epididymis and supply ATP (adenosine-5'-triphosphate), that is essential for testicular development and spermatogenesis. As it was also described by Colagar *et al*. (2009) and Wong *et al.* (2001), calcium can bind to calmodulin; that regulates the activity of several enzymes which are related to the motility. Hence it has a key role in the progressive motility of sperm. As to the authors, manganese is also a component of a metalloenzyme that is

linked to the synthesis of steroids; as a result it increases release of gonadal hormones which in turn have great contribution for libido and good quality semen. Another crucial element in the physiology of spermatozoa is magnesium, which is present in high concentrations in semen and is required in almost all enzyme systems. This element has an important role in spermatogenesis, sperm motility and ejaculation (Colagar *et al.*, 2009; Wong *et al*., 2001).

The interval between two collections is known to significantly affect all semen quality traits in young and mature bulls. Too frequent semen collections results in incidence of high frequency of sperm cells with cytoplasmic droplet, which indicates an incomplete phase of sperm maturation in the epididymis. A smaller volume of ejaculate, sperm concentration and total number of spermatozoa per ejaculate are apparent with a shorter period between collections for both young and mature bulls.

In semen processing, extended semen is allowed to stand 3 to 4 hrs in the refrigerator for equilibration; after which the semen transferred from cold cabinet to a bio-freezer that gradually cools the semen to -140°C (Lemma, 2011). The survivability of the spermatozoa depends upon the optimum cooling rate. Optimal cooling rate reduces the excessive concentration of intracellular solutes and intracellular dehydration; thereby reducing extreme shrinkage of the sperm cell. Hence optimal freezing affects metabolic processes of the spermatozoa for long-term storage of the semen without bringing significant loss of fertility (Clulow *et al.,* 2008).

Although semen analysis may seem easy to perform, meticulous attention to details and techniques is essential in order to obtain accurate and reproducible analysis (Vincent *et al.*, 2012). Manual semen analysis using a light microscope has been the standard method for analysis in most semen processing centers (SPCs). However, manual analyses can be very subjective and prone to within and between technician errors.

There is no significance difference in sperm motility and plasma membrane integrity in extender containing streptomycin and penicillin (SP) vs combination of antibiotics:

gentamycin, tylosin, lincomycin and spectinomycin (GTLS) until the third day of storage at 5^0C (Akhter *et al.,* 2008). However, on fifth day of storage sperm motility and plasma membrane integrity were significantly better in extender containing SP compared with GTLS.

2.3. Semen Evaluation

The success of AI program largely depends on the quality of semen that has been rigorously evaluated by its gross and microscopic parameters (Demeke, 2010). It seems for this reason that Karolina *et el*. (2012) stated, the bull's frozen semen should contain not less than 40% of progressively motile and 80% of morphologically normal spermatozoa.

2.3.1. Conventional semen evaluation

Macroscopic Parameters: Semen quality parameters like color, volume, consistency and pH are some of the macroscopic assessments routinely evaluated by semen processing laboratories. Semen color, volume and consistency are observed visually with diagnostic value to a certain extent for functioning of accessory glands, possible sperm concentration and expected number of doses (Dhurvey *et al.,* 2012). Whereas the pH of semen can be measured either by using pH meter, pH paper or bromothymole blue (David, 2003).

Color of bull semen: it is a useful physical indicator in assessing the presence of foreign matters like puss, blood, feces, urine and any debris which can influence semen quality. The normal color of bull semen is usually whitish to creamy; though some bulls have been known to consistently produce yellow semen which is due to a harmless pigment known as riboflavin (Barszcz, 2012). Hence, nonconformity from such normal color can be an evidence for injury or pathology in the tract (Dhurvey *et al.,* 2012). According to the description of Barszcz (2012), pathological colors are pink/red suggesting the presence of blood (which appears as the result of penis abrasion, fistulas of cavernous bodies, urinary stones), green suggesting the presence of pus, yellow suggesting the

presence of urine, watery white suggesting lower quantity of spermatozoa or water that got to the semen while collecting using artificial vagina.

Volume of semen: the ejaculate volume is readily measured by collecting the sample directly into a graduated test tube. There is also an alternative technique to measure the volume of semen by weighing the tubes after semen collection on top-loading balance, and later converting the reading into milliliter by using a computer program. This latter has been known to reduce error associated with visual reading of the tube especially when small volume or bubbles are found (Bearden and Fuquay, 1997). Ejaculate volume, along with concentration and percentage of motile cells is known to be important in determining the dosage of the semen straw during semen processing (David, 2003).

In most of the time, the bull ejaculates range from 1 to 15 mL depending on the age, breed/genetic makeup of the bull. As it was described by Lemma and Shemsu (2015), semen volume is affected by both genetic and non-genetic factors; like age, breed, ejaculate frequency, nutritional status, geographic locations and seasons. According to Barszcz (2012), decreased quantity of ejaculate may also suggest lack of preparation of the bull or mistakes committed during collection. Moreover, as described before a change in this parameter is also usually resulted from diseases of the urogenital system. However, small volume is harmless, unless accompanied by a low spermatozoa concentration.

*PH***:** The pH of bull semen is slightly acidic; about 6.2 to 6.8 (Barszcz, 2012). It has been also reported to rise ($\geq$7) when there is incomplete ejaculation, excessive use of the bull or in yearling bulls and in pathologic situation of the testis, epididymis, ampulla or seminal vesicle (Sori, 2004). Moreover, dense semen samples which possess excellent motility have been known to show lower values of pH (Barszcz, 2012).

Microscopic Parameters***:*** mass activity, individual spermatozoa motility, viability and morphological evaluations are the parameters which are commonly practiced for conventional semen evaluation.

Mass activity: The mass activity of spermatozoa mimics the ability of the sperms to swim in the reproductive tract of cow once inseminated either artificially or naturally (Lemma and Shemsu, 2015). As it was described in Dhurvey *et al. (*2012) it is the most influenced parameter in the semen analysis if not examined soon. In assessing its scale, different authors categorize it in different ways; for instance Sori (2004) and Barszcz (2012) graded its scale as 0 to 5; Where 0, 1, 2, 3, 4, and 5 have been known to represent immotile sperm, stationary bunting (weak rotatory movements), oscillatory movements with no wave motions or eddies, motions with slowly/moderately moving waves and eddies, spermatozoa movements exhibiting rapid waves and eddies and extremely rapid waves and eddies respectively. And as to Barszcz (2012), the semen used in AI should be characterized by at least moderate waving progression. On the other hand, Lemma and Shemsu (2015) graded as 0 - 4 scales (0 = no mass activity; 1 = weak mass activity without forming any wave; 2 = small, slow moving wave; 3 = vigorous movement with moderate rapid waves and eddies and 4 = dense, very rapidly moving waves and eddies).

*Individual Motility***:** The motility of sperm cell is further evaluated on an individual basis; which has been defined as the percentage of individual sperm cells that are motile individually in the absence of any external influence. Sperm cells moving straight-line in a forward direction are considered to be progressively motile (Bearden and Fuquay, 1997). Even though microscopic sperm motility testing is the most commonly used parameter to determine the quality of semen intended for AI; the routine conventional motility assessment is completely subjective and depends on the experience and ability of the person performing the test. Bull spermatozoa motility below 40% or 50% has been known to be associated with low conception rate or poor fertility (Bearden and Fuquay 1997; David, 2003).

Live-Dead Ratio: as it provides a check on the accuracy of individual motility estimation (hence percentage of dead cells has to be less than percentage of immotile spermatozoa); conducting vital stains like Eosin-Nigrosin are paramount important in quantifying the fraction of live and dead cells (Dhurvey *et al*., 2012). Under differential staining/Eosin-Nigrosin; dead spermatozoa stain red with eosin against the dark nigrosin background

while the live cells exclude the stain (Salisbury *et al*., 1978). The accepted minimum standard percentage of live spermatozoa before freezing in bull semen collected by artificial vagina which intended to be used for AI is 70% (David, 2003). Higher percentages of dead sperms may be attributed to diseases, age or dysfunction of accessory glands/testes. Moreover as immature spermatozoa have a short functional life; this condition can also contribute for increased number of dead cell counts. The cryopreservation process is also known to reduce sperm viability by 50-60% (Lemma, 2011).

Sperm Morphology: On account of the fact that freezing and thawing process provokes morphological or biochemical cryogenic damage resulting in sperm dysfunction and changes in cell's membrane; the sperm morphology evaluation is an essential component of any semen analysis and hence it provides the clinical information about the potential fertility of semen sample (Lemma, 2011).

Sperm has basically three functional regions: a head that contains the condensed nuclear material, a mid-piece serving as a power house and a tail which is the propulsive region. However, there are probabilities that the morphology of the sperm from an individual bull may deviate from the normal, these may be attributed to the genetics, disease and age of the bull, (young and old bulls tend to produce sperms, which may have morphological problems). As suggested by Barszcz (2012), total number of morphologically altered spermatozoa in fertile bulls should not exceed 15%. The major morphological abnormalities usually occurred are detachment of head, bending of the middle piece and/or tail, tailless sperms and coiled tails (Bearden *et al.*, 2004). Detached or loose heads have been known to be abnormalities which possibly could occur due to excessive agitation or incorrect smearing of spermatozoa; whereas, high percentages of middle piece protoplasmic droplets have been observed in over used bulls due to rapid passage of spermatozoa through the epididymis (David, 2003). Cold-shock due to the presence of water or urine in the ejaculate has been noted to cause bending of the tail at the junction of the tail and middle piece with or without a protoplasmic droplet (David, 2003).

According to this author, abnormalities of middle piece and tail have been reported to interfere with fertility by reducing sperm motility.

Concentration: it can be determined via a number of methods like hemocytometer/ Neubar chamber, spectrophotometer, computer aided microscopy, and fluorescent assay techniques. However, because of its little more tediousness; Neubar chamber for estimating sperm concentration has been skipped by most technicians to test completely. For this reason spectrophotometer is mostly practiced to determine the concentration of sperm cells. With spectrophotometer, a beam of light passing through the sample is measured using the phototube which is inversely correlated to the sperm concentration/ml (Rai *et al.* (2017),. Even though, it is preferred instead of Neubar chamber test for its being unsophisticated; it has its own drawbacks in differentiating dead and live sperm cells while giving approximate counts. therefore, to get the required population of progressively motile live sperm cells per dose, one has to consider other tests like live:dead ratio and motility percentage together with concentration evaluation as it was suggested by Dhurvey *et al*. (2012). As a result, as described in Brito *et al*. (2002) the total number of sperm cells per ejaculate can be calculated by multiplying the concentration with the volume of ejaculate. In the same way the total count should be multiplied by progressively forward moving percentage (individual motility) to get the total count of progressively motile spermatozoa. All these (ejaculate volume, total count and progressively motile spermatozoa) together with number of spermatozoa per straw to be packed can be the bases for calculating the total dose produced per ejaculate. Determination of the sperm concentration is necessary not only to alleviate the likelihood existing variation in number of sperm cells between batches but also for the quantification of those cells required for optimum fertility. An average of 1.2×10^9 sperm cells/mL (with the range is from 0.3 to 2.5×10^9 sperm cells / mL) is expected in the bull ejaculate. Apart from breed differences, minor variations in concentration may exist between the different techniques like haemocytometer, spectrophotometer and electronic counting systems (Zrimsek, 2011).

2.3.2. Functional tests

Hypo osmotic swelling test /HOST/: In most semen producing laboratories, conventional semen evaluation is conducted on the basis of ejaculate volume, color, spermatozoa concentration, motility, morphology and viability. However, as the fertilization process comprises many physiological events; such conventional subjective semen evolution methods lack efficiency to predict the fertilizing potential of male animals. On the other hand, as it was described by Petrunkina *et al*. (2007), hypo osmotic swelling test superiorly correlated with the AI outcome than the results of routine conventional semen evaluation tests. This high association of HOST with other fertility predictive test is also supported by pregnancy rates in pigs (Pérez-Llano *et al.,* 2001).

Freezing and cryopreservation of bull semen is the prerequisite to avail the semen for a wide range of area and use it for longer time period in the area where AI is practiced. Moreover, an intact and functionally active membrane is essential for the spermatozoon to undergo appropriate metabolism, capacitation, acrosome reaction and for further attachment and penetration of the oocyte zona pellucida (Dhurvey *et al.,* 2012). However, as the temperature decreases due to cryopreservation process the spermatozoa undergo lipid phase transition from the fluid phase to the gel phase and this drastically reduces semen quality by destabilizing the lipid architecture of plasma membrane. As a result, affected spermatozoa are predisposed for premature capacitation and spontaneous acrosome reaction which in turn leads to reduced ability to bind and penetrate the zona pellucida (Thundathil *et al.,* 2000; He *et al*., 2001). Moreover as stated by Thundathil *et al.* (2000), though membrane cholesterol has a stabilizing effect on spermatozoa membrane; any change in its content also induces reorganization or destabilization of the membrane architecture. Due to the significant importance of plasma membrane in the process of fertilization and for its being the primary site where lesions occur during freezing and thawing, evaluation of its integrity has got significant attention in predicting the male fertility potential (Zubair *et al.,* 2013).

Though charged dyes like trypan blue, propidium iodide (PI) and eosine-nigrosine selectively can label dead cells and are used to differentiate live and dead sperm cells; such evaluation tests could not measure the live spermatozoa plasma membrane functional activity (Curry and Watson, 1994). To obtain more information about the reproductive physiology of plasma membrane of spermatozoa, HOS test was proposed as a humble method to assess the structural and functional membrane integrity and activity of spermatozoa. However, it is not recommendable to use HOST as the only fertility test of sperm (Brito *et al.,* 2002); but can be an additional vitality test and hence it supplements the information provided by the conventional semen evaluation parameters used to assess fertilizing ability of the sperm. Hypo osmotic swelling test is a simple, inexpensive and easily applicable technique, which has been confirmed and standardized as a good tool for evaluating the sperm membrane integrity and functional activity of various species: bovine (Rota *et al.,* 2000), goat (Fonseca *et al*., 2005), pig (Perez-Llano *et al*., 2001; Samardzija *et al.,* 2006) and stallion (Nie and Wenzel, 2001; Pinto and Kozink, 2008).

Functional integrity of the plasma membrane can be evaluated based on the ability of the membrane to allow passage of water in order to establish equilibrium between the fluid compartment within the spermatozoon and the external surroundings after incubation for 60 minutes at 37ºC (Neild *et al.*, 1999). Functionally active spermatozoa exposed to a hypo-osmotic stress swell due to the influx of water and subsequently increase in volume to establish the equilibrium between the cytosol and the extracellular milieu. In contrast, spermatozoa with compromised or functionally inactivated membranes are unable to regulate water influx and allow fluid to move freely across the membrane without any accumulation inside; hence no cytoplasmic swelling and curling of the tail would occur. Thus, hypo-osmotic swelling test is useful in assessing changes of sperm membrane functional integrity (Dhurvey *et al.*, 2012).

According to Colenbrander and Kemp (2003), HOST seems to be more appropriate for predicting the fertilizing capacity of frozen-thawed than fresh semen, because membrane damage in frozen-thawed semen is a more important limiting factor than in fresh semen.

As it was described in Mishra *et al.* (2013), hypo osmotic swelling reactivity of spermatozoa varies among seasons. According to the authors, significantly decreased HOST reactive spermatozoa percentages was recorded for Red Sindhi, Crossbred, Haryana and Jersey breeds with the increase of ambient temperature. In contrast, Barth and Waldner (2002) and Koonjaenak *et al.* (2005) reported highest percentage of HOST reactive spermatozoa percentage during the summer season in buffalo. However, as it was described by Koonjaenak *et al.* (2005), such differences could have been also as a result of variation in sheltering and management.

Acrosome Integrity: Acrosome is a cap like structure covering the front side of spermatozoon nucleus, and it is bounded by the membrane which contains a specific lipoprotein complex, including enzymes like hyaluronidase, acrosin, esterase, acid hydrolase, and other proteinases which are known to be involved in the fertilization process (Thundathil *et al.,* 2000).

Spermatozoa capacitation and the subsequent acrosome reaction are the essential steps conducted before fertilization and zygote formation (Chowdhury *et al.,* 2014). Hence, though not the only factor; maintenance of optimum fertility depends on the acrosome being structurally and biochemically intact, which contains the enzymes necessary to penetrate through the outer layers of the ovum and achieve fertilization (Thundathil *et al.*, 2000). In normal circumstance, it is the conjunction force of spermatozoa with ovum that releases the acrosome ingredient to the surface of spermatozoa, which in turn soften and melt cumulus cell matrix (hyaluronidase enzyme) and plays a role in passing of the sperm cell through the zona pellucida (acrosine); and makes penetration of this structure becomes easier for the spermatozoon (Neild *et al*., 2005; Witte and Schafer-Somi, 2007). However, when these acrosomal ingredients are not maintained until conjunction with ovum; the expected fertility potential of the spermatozoa drastically decreases.

Acrosomal status is mainly assessed by using plant lectins recognizing acrosomal ligands (Vincent *et al.,* 2012). *Pivum sativum agglutinin* (PSA) derived from the pea plant and *Arachis hypogaea agglutinin* (PNA) derived from the peanut plant are the most

commonly used plant lectins recognizing acrosomal ligands (Dhurvey *et al*., 2012; Vincent *et al.*, 2012). According to the authors *Pivum sativum agglutinin* cannot penetrate the intact acrosomal membrane; and only binds and stains to mannose and galactose moieties of the acrosomal matrix of damaged acrosome. However, *Pivum sativum agglutinin* has also an affinity for egg yolk and non-specific binding sites on the sperm cell surface (Lybaert *et al*., 2009) and this could become a problem and resulting misinterpretation of the acrosomal status when analyzing semen diluted with egg yolk-based extender. *Arachis hypogaea agglutinin* binds to galactose moieties of the outer acrosome membrane and is the most popular lectin used to study the integrity of the acrosomal membrane with flow cytometry (Vincent *et al.*, 2012). According to the authors it also displays less for other non-specific binding areas of spermatozoa. Spermatozoa with reacted, damaged, or abnormally formed acrosome acquire green fluorescence after PNA labeling, while intact, normal acrosome have no fluorescence (Dhurvey *et al.* (2012). Giemsa stain is also another recommended method for the assessment of acrosome integrity (Chowdhury *et al.*, 2014). It only binds to the outer intact acrosomal membrane; but not to the damaged or acrosome-reacted once.

1.3.3. Advanced semen evaluation

1.3.3.1. Computer assisted semen analysis (CASA)

Sperm cell comprises complex structures that are important for successful fertilization of the ovum (Dhurvey *et al.*, 2012). In addition to its structural complexity; it is also difficult to obtain accurate prediction about its fertility potential using conventional subjective semen evaluation methods. Studies (Bochenek *et al*., 2001; Vincent *et al.*, 2012) have shown that these technical inaccuracies of subjective semen evaluation can be reduced when objective methods are used. Now days, computer assisted semen analyzer is one of the automated machines developed to evaluate various characteristics at individual cell level.

Computer assisted semen analysis was developed at the beginning of 1980s, aimed at providing a more objective analysis of sperm motility by reconstruction of individual spermatozoa trajectories for semen quality assessment and fertility estimation Galmessa *et al*., 2014). As it was described by different authors (WHO, 2010; Agnieszka *et al.*, 2012; Ratnawati and Luthfi, 2020) CASA is one of the choices of techniques/ methods for evaluating semen quality and is a powerful tool for the objective evaluation of spermatozoa concentration, individual cell motility percentage, motion characteristics, kinematics, morphology and viability.

The basic components of CASA are a microscope to visualize the sample, a digital camera to capture images and a computer with its specialized software to analyze the movement of the spermatozoa (Vincent *et al.,* 2012). According to these authors and Holt *et al.* (2007), the essential principle behind microscopy-based CASA systems is that a series of successive images of motile spermatozoa within a static field of vision are acquired by computer software algorithms, which then scan the image sequences to identify individual spermatozoa and trace their progression across the field of vision; Viz in each image individual spermatozoon is recognized based on its position in the field, and the subsequent next position of each cell is again inferred by estimating the likelihood that it will have moved a maximum distance between frames; then the respective data obtained are mathematically processed, and Lastly, trajectories are defined in numerical form and the results from this operation are reflected as a series of parameters which accurately define the motion of each spermatic cell.

1.3.3.2. Description of CASA parameters

Evaluation of spermatozoa motion characteristics by CASA system provides a wide range of kinematic parameters which have high informative value in characterizing the physiological state and possible fertilizing ability of spermatozoa (Kathiravan *et al.*, 2011). Percent total motility (MOT), progressive motility (PMOT), curvilinear velocity (VCL), average path velocity (VAP), straight line velocity (VSL), amplitude of lateral head displacement (ALHD), Linearity (LIN), straightness (STR), beat cross frequency

(BCF) and mean angular displacement (MAD) are some of the major parameters those can be assessed by CASA system (Niżański *et al*., 2009; Agnieszka *et al.,* 2012).

Table 1. Sperm kinematic indexes for approval of bovine semen by ISAS

Sperm kinematic index	Minimum value for approval by ISAS
VCL	10μm/s
VCL of <10μm/s	Static
VCL of 10- 25μm/s	Slow
VCL of 25 - 50μm/s	Medium
VCL of >50μm/s	Rapid
VCL of 25 – 50μm/s and STR > 70%	medium progressive motile sperm
VCL of >50 μm/s and STR > 70%	rapidly progressive motile sperm
Progressivity	STR >70%

Source: Manufacturer of integrated semen analysis system (ISAS® PBos, PROISER - Projectes i Serveis R+D S.L., Spain).

Other functional kinematic parameters (VAP, SLV, STR, LIN) can be computed indirectly based on the individual spermatozoon VCL trajectories by the ISAS software program itself.

Percent Motility (MOT): is defined as the number of motile cells divided by the total number of cells analyzed and expressed in percent (Dhurvey *et al.,* 2012). According to Sundararaman *et al. (*2012), a bull spermatozoon is considered to be motile if its average VSL met or exceeded the minimum (4.4μm/s) motile speed. Similarly, to consider a bull sperm as motile; the manufacturer of integrated semen analysis system (ISAS® PROISER., Spain) also recommended a VCL of at least 10μm/s. Additionally, Dhurvey *et al*. (2012) recommended a consideration of at least 200 cells for analysis to express the sperm cell motility percentage and get reliable results.

Progressive Motility (PMOT): the population of cells in percent those are moving actively in a forward direction (Agnieszka *et al.*, 2012). A progressively motile sperm is defined as the one which has VAP > 50 µm/s and a straightness ratio (STR) > 75% Paul (2013). Likewise, the manufacturer of ISAS also suggested a minimum VCL of 25 µm/s and STR > 70% to consider as medium progressive motile cell and a minimum VCL of 50 µm/s and STR > 70% to consider as rapidly progressive motile cell.

Curvilinear Velocity (VCL): this is measured in unit of µm/s and defined as time-average velocity of a sperm head along its actual curvilinear path while it is perceived in two dimensions under the microscope. It is a measure of cell vigor. It is computed only for motile cells and is achieved by averaging the mean values from each individual cell.

Average Path Velocity (VAP): As it was defined by Agnieszka *et al.* (2012) and Galmessa *et al.* (2014), average path velocity is computed by smoothing the actual path and used to characterize the overall trajectory of the sperm cell and is also measured in unit of µm/s. For the reason, this path is computed by smoothing the curvilinear trajectory according to the algorithms in the CASA instrument; it is not recommendable to compare values among systems when variation for algorithm exists between systems (WHO, 2010).

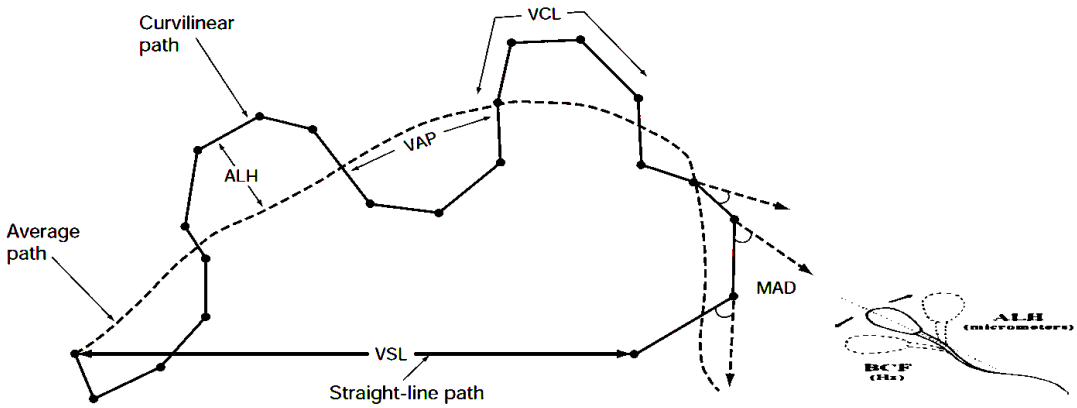


Figure 1: Schemes of different velocities and parameters of sperm movements while measured by CASA system.

(Restructured from WHO, 2010 and Agnieszka *et al.*, 2012)

Straight Line Velocity (VSL): this is also measured in μm/s and defined as the average velocity measured in a straight line from the beginning to the end of the track. It is a measure of cell's forward progression that can be computed by multiplying the curvilinear velocity (VCL) with mean linearity divided by 100. Like that of VCL and VAP; It is also computed for the population of motile cells by averaging the mean values of individual cell (Dhurvey *et al.*, 2012).

Linearity (LIN): The distance a sperm cell travels along its normal (un-smoothed) path is referred to as its gross displacement. The straight line distance from its starting point to its current X-Y position (as the crow flies) is referred to as net displacement; and based on these variables Agnieszka *et al.* (2012) defined it as the ratio of these two measures multiplied by 100 (i.e VSL*100/VCL) which is expressed in percent. It is evaluated at the end of each of the motile paths and all of the motile cell path values are averaged to get the single number for the report. A cell that swim in a straight line has value of 100% and a cell that had just completed a circle had an instantaneous value of zero (Galmessa *et al.*, 2014).

Straightness (STR): Straightness is a measure of VCL side to side movement and determined by the ratio of (VSL/VAP) multiplied by 100 and like that of linearity this also measured in unit of percentage (Agnieszka *et al.*, 2012).

Amplitude of lateral head displacement (ALH): For each cell, the distances between the actual curvilinear and the smooth (or average) path are computed. These values are sometimes referred to as RISERS. This parameter (ALH) is computed by the maximum value of the RISER in μm for each path and then computed as the average value of all of the individual maxima as the single value to include in the report of lateral head displacement of motile cell population (Dhurvey *et al.*, 2012). Agnieszka *et al.* (2012) simply defined this parameter as the mean width of the head oscillation while the sperm cell moves. However different CASA instruments compute ALH using different algorithms, and hence values may not be comparable (WHO, 2010).

Beat cross frequency (BCF): It is measured in Hz and Agnieszka *et al.*, (2012) defined this parameter as the frequency with which the sperm head moves back and forth in its track across the cell path.

Wobble (WOB): A measure of oscillation, determined by the ratio of (VAP/VCL) multiplied by 100 and like that of linearity and straightness this parameter is also measured in unit of percent (WHO, 2010).

Mean Angular Displacement (MAD): It is the time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory measured in degrees (WHO, 2010)

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1.3.3.3. Attributes measured by CASA

Motility: It is one of the most important characteristics believed to be associated with the fertilizing ability of spermatozoa. A significant correlation between total (Gillan *et al.*, 2005) and progressive (Kathiravan *et al*., 2008) motility of bull semen with field fertility had been previously reported. Researchers have also tried to correlate the kinetic parameters with the field fertility of semen. A positive correlation between STR of spermatozoa and field fertility has been reported by various authors (Farrell *et al*., 1995; Januskauskas *et al*., 1999; Gillan *et al.*, 2005; Kathiravan *et al*., 2008). However, as it was described by different researchers (Ahmadi and Soon-Chye, 2003; Dhurvey *et al.*, 2012), this also become true and very high correlation of bull fertility have been reached through combined several motility parameter evaluations.

Morphology: On account of the fact that freezing and thawing process provokes morphological or biochemical cryogenic damage; sperm dysfunction and changes in cell's membrane are the effects to be observed. For this reason the sperm morphology evaluation is an essential component of any semen analysis and provides the clinical information about the potential fertility of semen sample. Furthermore, while undertaking

sperm morphological evaluation, sperm head morphology is an important criterion for the correct diagnosis of sperm quality (Ahmadi and Soon-Chye, 2003).

According to WHO (2010), the CASA software set up is used to analyze images of sperm smear stained with Diff-Quik stain. The Diff-Quik dye stained pale blue in the acrosomal region and dark blue in the post-acrosomal region of sperm cell which is a good base for precise image analysis. As a result, by using CASA; morphology of the head parameters such as area of the head, perimeter of the head, elongation of the sperm head (Elng), form factor circle: the degree of similarity of the sperm head to a circle (FFC), Big axis of ellipse-outlining the sperm head, the length of the sperm head (ELL_B), Small axis of ellipse-outlining the sperm head, the width of the sperm head (ELL_S) and form factor ellipse-the degree of similarity of the sperm head to an ellipse (FFE) can be assessed.

Like Diff-Quik stain a number of stain methods have been suggested for sperm morphological assessment; however, some of the stain-methods are certainly species-dependent and do not necessarily provide the appropriate gray-level contrast for accurate computer-assisted morphometric analysis. Papanicolaou's staining and haematoxylin are the two morphologic assessment stain methods mostly used for sperm morphological evaluation using CASA (WHO, 2010). This was also supported by Ageep *et al.* (2009), that discovered a cumulative percentage of more than 86% for both excellent and very good grading at spermatozoon head region using the two (Papanicolaou and Haematoxylin) stains in comparison to other four stains (Supravital, MGG, Giemsa and Leishman) through result interpretation grade of excellent, very good, good, bad and very bad for evaluation of spermatozoon parts.

Viability: CASA is also important in identifying sperm viability and differentiation of sperm cells from debris materials under the application of different stain dyes (Dhurvey *et al*., 2012). According to the authors, it determines the sperm viability by using a vital stain- VIADENT'. This dye stains only the cells with non-intact membranes; thus identifying non-viable from viable cells. CASA also differentiates sperm cells from debris using IDENT stain under fluorescent illumination. This is a DNA specific dye that can stain all DNA containing objects and fluoresce under appropriate light; thus

differentiating sperm cells from debris materials. As stated by Contri *et al*. (2010), cytoplasmic detritus, which is devoid of DNA material, have lower degree of fluorescent intensity than the haploid sperm and these may not fluoresce while examination. Interestingly, according to these authors, although sperm cells have only half the DNA complement of a somatic cell, it is highly condensed, and results a higher degree of fluorescent intensity

1.3.3.4. Factors affecting CASA results

Although CASA systems have been demonstrated to possess higher accuracy than the traditional methods (Didion, 2008; Valverde *et al.,* 2020), the final results of the semen samples can be influenced by several factors, such as dilution of the semen samples, time elapsed since ejaculation until examination, sample temperature, counting chamber type and depth (Del Gallego *et al.,* 2017), evaluation algorithms and number of analyzed fields (Broekhuijse *et al.,* 2011), recording frame rate (Valverde *et al.,* 2019), type of CASA system, system parameter and threshold settings (Boryshpolets *et al.,* 2013) and other numerous factors like concentration of spermatozoa, percent motility, temperature at which semen is analyzed and type of extender used for dilution. Nevertheless, reliable and reproducible results can be obtained if appropriate procedures are followed.

Frame rate: The video camera that determines the frame rate of the system could be the potential source for the result variation; hence, various models of video camera which are continuously evolving in the semen evaluation have different frame rates. The studies of Brito (2010) and Castellini *et al*. (2011) have shown the importance of the frame rate for reliability of the analysis. As spermatozoon motion characteristics is defined by several kinematic parameters, like VCL which are very sensitive to the lapse between images capture; motility is affected by frame rate of sequence capture. Most of the CASA systems allow 30 or 60 Hz as a frame rate to analyze sperm tracks and speed. According to Valverde *et al.* (2019), a higher frame rate is required to render evaluation closer to the real path for a fast non-linear sperm cell; and frame rate of 60 fps is good for bull semen

analysis. According to the authors, though some disagreements had been existed regarding the time for which spermatozoa should be followed to achieve accurate results; most recommends a minimum of 1 second for the basic CASA measurements.

Digitization Threshold: For some years after the development of CASA technology; digitization threshold were been one of the factors affecting CASA results. Boryshpolets *et al.* (2013) have shown the significant effect of image digitization threshold on CASA results. Accuracy of sperm recognition and tracking are the two parameters those mainly affected by digitization threshold.

*Specimen Concentration***:** The CASA instrument inability to obtain accurate counts and percent motilities when the concentrations of the specimen is greater than 50 million or less than 20 million per ml and when the specimen is laden with debris are fundamental limitations (Valverde *et al.,* 2020). This idea is also supported by Contri *et al*. (2010) that stated, at low sperm concentration (less than 20 million per ml), an over estimation of the concentration and thus underestimation of the percentage of motile cells can occur due to the acquisition of non-spermatic particles (debris) and at a higher sperm concentration (above 50 million per ml) a large proportion of the fast moving cells excluded from analysis because of their exit from the analysis area. Moreover, at a higher sperm concentration, collision of cells for moving spermatozoa also the other challenge disturbing the motion characteristics leading to underestimation of the motility percentages and low values of kinematics. For this reason laboratories have to be required either to concentrate or dilute specimens which in turn severely limit the routine clinical application of the technology. Thus accuracy and precision of concentration, motility and kinematic variables are the most potentially affected parameters while analysis is done by CASA (Valverde *et al.,* 2020).

Percent motility: the inaccuracies of CASA for percent motility have been due to the error for overall sperm count and biasness to classify motile and immotile cells (Contri *et al*., 2010). Besides to this, the definition of motile sperm for subjective visual evaluation and CASA approaches is not the same; in visual analysis, a spermatozoon is considered

motile if its flagellum is twitching, even though it may have no forward progression whereas in CASA a spermatozoon must achieve a minimum VSL to be considered as motile. For instance Sundararaman *et al*. (2012) set 4.4µm/s for a spermatozoon to be considered as motile and hence, CASA measured motility percentage values will usually be lower than subjective visually estimated percentages.

Specimen Chamber Type and Depth: the type of specimen chamber used for analysis can affect the motion characteristics of sperm, the accuracy of the cell count and percent of motile spermatozoa (Massányi *et al*., 2008). In agreement to this idea; Lenz *et al*. (2010) also reported a difference in values of different parameters among two specimen chamber types (2-cell and 4-cell leja slide). For proper analysis of sperm concentration using CASA; the use of well-defined counting chamber that fulfills rheologic and capillary forces for designing plane movement and best distribution of spermatozoa is desirable. The question also arise for motility analysis, taking into account that the cells are forced to move in a plane. For a cell having about 70 µm in length, movement in a space of only 10 µm of height produces a clear motility disruption; hence, depending on the species, chambers of 20 µm reduces this effect, even it doesn't disappear.

Temperature: the temperature at which semen is analyzed is also another factor that can affect CASA results. Movement of sperm, particularly motility percentages and curvilinear velocity are the two most potentially affected parameters by temperature. In supporting this Vincent *et al*. (2012), demonstrated the decrease of motility parameters (percentage of motile spermatozoa and track speed) when spermatozoa are not analyzed at a temperature of 37^{o}C.

*Type of Extender**:*** Some extenders contain debris of size similar to a sperm head, causing CASA software to include them in the analysis. Egg-yolk and milk based diluents are examples of extenders containing such particles (Davis and Katz, 1992). In addition, when observing semen diluted with milk extender, the globular lipids mask the spermatozoa. Thus as it affects many of the kinematic parameters, the type of extender in

which semen is diluted is also another aspect that should be taken into consideration when evaluating spermatozoa with CASA.

Table 2. Other possible factors that affect CASA results

Factor	Effect
Microscope optics and illumination methods	Accuracy of image digitization, target recognition and tracking
Laboratory supplies	Can be cytotoxic
Drop-to-drop variability	Accuracy and precision of all measures
Physiological state of the sperm	Rapidly swimming or capacitated sperm require a significantly higher video sampling rate to obtain accurate results

Adapted from Didion (2008) and Valverde *et al.* (2020)

Chapter III

MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted at Livestock Development Institute (LDI) situated at Akaki-kality sub-city of Addis Ababa. The city lies at 9°1' North latitude, 38°44' East longitude and the altitude ranges from 2000 - 3000 meters above sea level (Sori, 2004). The annual rain fall of the city ranges from 750 - 1500 millimeters and its average daily temperature is about 16°C (National Meteorology Agency; https://www.weather-atlas.com/en/ethiopia/addis-ababa-climate). The former National Artificial Insemination Center (NAIC) later named National Animal Genetic Improvement Institute (NAGII); and more recently renamed as Livestock Development Institute (LDI) was established in 1981 and is a governmental AI service provider organization in Ethiopia through regional agricultural offices. The institute is also responsible for importing bulls used for semen production and in-calf heifers those can serve as dams for further candidate breeding bulls. Moreover, by considering their special merit of interest, the institute also selects local breed of bulls for semen production.

3.2. Sample Size and Sampling Procedures

A total of 14 (Holstein Frisian/HF/ = 6, Boran*HF Crosses = 4 and Boran = 4) bulls were considered for this study from 51 breeding bulls (Holstein-Friesian = 20, Jersey = 16, Boran*HF Crosses = 9, Boran = 4, Erob = 1 and Sheko = 1) found at LDI. Study bulls of HF and Crosses were randomly selected for the respective breed by using their ID for lottery system; whereas all Boran bulls were included due to their small number. The bulls were aged between 2 and 7 years. They were all kept indoor under identical conditions of management, feeding and watering throughout the study period. They received hay, green forage and concentrate fortified with minerals. Water was given *ad libitum*. They were also allowed to exercise on running track on a weekly basis.

3.3. Data Collection

3.3.1. Breeding soundness evaluation

Breeding soundness evaluation to assess the breeding potential of bulls was conducted as it was described by Lemma and Shemsu (2015) with the considerations of their history and general physical examinations (eyes, legs, feet, etc.) along with careful examinations of the reproductive tracts and semen quality. Accordingly, to evaluate their implication on fertility; libido, genital system integrity particularly the testes for their size, symmetry, form, consistency and movability inside the scrotal sac were considered and assessed after restraining each bull in the chute.

Testicular parameters (length, width, thickness and scrotal circumference) were measured using caliper and flexible tape to the nearest 0.1 value according to the method suggested by Barth (1997). Testicular symmetry, form, consistency and movability inside the scrotal sac were evaluated by palpation and bulls with testes of firm consistency that were movable within the scrotal sac and appeared symmetrical, passed to the next level of breeding soundness evaluation.

Functional testicular parameters (volume and weight) were driven from the testicular measurable parameters (length, width and thickness) using a mathematical volume formula for prolate spheroid and bovine testicular tissue density as described below.

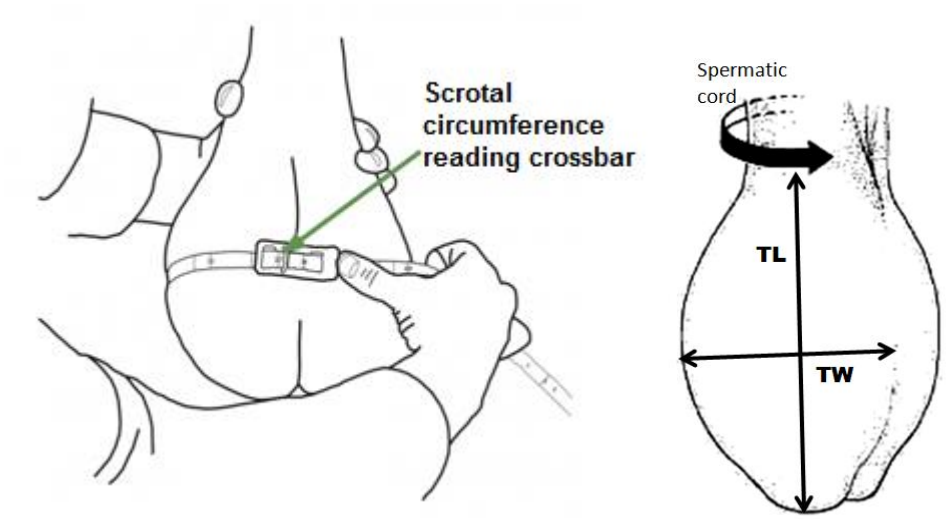


Figure 2. Testicular measurements

Volume of *Testes:* volume of testes was calculated using a mathematical formula for prolate spheroid volume described by (Bailey *et al.,* 1997).

$$V = \frac{4(\pi abc)}{3};$$ Where: a = thickness/2, b = width/2 and c = length/2.

Weight of the Testes: this was also mathematically calculated from the product of each testis volume (Bailey *et al.,* 1997) with expected bovine testicular tissue density (1.038) investigated for cattle (Amann, 1990).

$$W = 1.038(V);$$ Where: W = testis weight and V = testis volume.

Accessory Sex Glands: The accessory sex glands (seminal vesicle, Prostate and bulbourethral) were assessed for their development upon rectal palpation, and bulls with accessory sex glands of well-developed passed to the next level of breeding soundness evaluation.

Libido: was evaluated based on 1-4 scales (1- shy or has no desire to move towards a teaser, 2- dull or very reluctant to reach the teaser, 3- active or willingly moves towards the teaser and 4- aggressive or moves towards teaser in an uncontrolled manner).

3.3.2. Semen collection, processing and evaluation

A total of 125 ejaculates (57 from HF, 33 from Boran*HF Crosses and 35 from Boran) were collected on a weekly basis for evaluation. Bulls were given bath to remove dirt from their prepuce 30 min before collection of semen. Semen was collected using bovine artificial vagina (IMV; France) early in the morning between 09:00 - 11:00 AM as per the method described by Zubair *et al.* (2013) which was also the routine practice at LDI. Immediately after collection, semen was grossly assessed for its volume, color, pH and presence of contaminants such as blood, puss, hair and other debris. It was then placed in a water bath set at 37 ^{0}C until further microscopic evaluation. During the macroscopic and microscopic semen quality assessment; ejaculates which didn't fulfill minimum criteria standards of the laboratory (semen contaminated with urine and/or blood, semen volume < 2ml, semen with spermatozoa concentration < 0.5 billion/ml, semen with wave motion intensity for mass activity < 3, initial individual spermatozoa motility < 70% and post freeze individual spermatozoa motility < 40%) were removed from production and their corresponding data also were not considered for the analysis. Ejaculates were diluted using OptiXcell extender (IMV Technologies; France) to attain a final concentration of 142.86 million spermatozoa/ml; after which they were filled and sealed into labeled mini straws (IMV Technologies; France). The semen in the straws were distributed on rank in a horizontal position and allowed to equilibrate at 4°C for about 4 hours and then shifted to a programmed bio-freezer (freezing curve: 5°C to -10°C at 5°C/min; -10°C to -110°C at 40 °C/min and -110°C to -140°C at 20°C/min) where the temperature was brought down to -140°C within 7 minutes using liquid nitrogen vapor. Lastly, the straws were shifted to periodically top upped liquid nitrogen containers for storage till distribution and use at field level.

Conventional Semen Evaluation: during the macroscopic assessments, volume of the ejaculate was recorded from the graduated tube to the nearest 0.1 ml. Semen pH was also measured to the nearest two decimal values using pH meter. Color of the semen was classified as creamy, milky, yellowish, and watery. Microscopic evaluations of mass activity (score), individual progressive motility (%), morphology and live percent were conducted according to procedures described in Lemma and Shemsu (2015).

*Motility (Mass activity and individual progressive motility)***:** mass activity was evaluated by placing 5µl of undiluted semen on pre warmed slide and heated stage microscope (thermostatically set at 37^0C) with a magnification of 100X and scored based on its intensity of wave motion on 0 - 4 scales (0 = no mass activity; 1 = weak mass activity without forming wave; 2 = small, slow moving wave; 3 = vigorous movement with moderate rapid waves and eddies and 4 = dense, very rapidly moving waves and eddies). In the meantime, fresh individual progressive motility was also estimated on phase contrast microscope (ZEISS ECLIPSE E400, Tokyo, Japan) at a magnification of 200X and scored 0 to 100% based on estimation of percentage of spermatozoa which progressively move in a forward direction. Chilled and frozen samples from 0.25ml straws of the same batch were similarly evaluated after thawing the samples in a water bath at 37°C for 30 s.

Live:Dead Spermatozoa Ratio: viability of sperm cells was evaluated using eosin-nigrosine staining as per the method described by Bjorndahl *et al.* (2003). Briefly; microscope slides and eosin-nigrosin stain (1.67gm of eosin and 10gm of nigrosin in 100 ml distilled water) were pre-warmed at 37^oC. The mixture of the stain (15µl) was pipetted onto the edge of a slide followed by 5µl of frozen-thawed semen loaded next to the stain. The stain and semen were mixed and then smeared on the surface of the slide. The slide was blow dried and examined using a bright field microscope (40X objective lens). Unstained sperm cells that appeared white were considered as live and those absorbing the eosin and appeared pinkish in color (as a result of lost membrane integrity) were considered as "dead". In each observation, at least 200 sperm cells were counted to compute the live:dead ratio.

Morphological Evaluation: this was conducted as described in Freneau *et al.* (2010) for differential interference phase contrast (DIC) microscopy of wet-mount semen fixed in isotonic formal saline. Procedurally, 1ml of Hancock solution (Buffered formole saline) was kept at 37^0C in water bath and fresh semen sample of 5µl was added with pre warmed (37^0C) Pasteur pipette tip and then gently mixed for examination. Morphological defects were regionally classified as head (detached head, small abnormal head, Pear shaped head, acrosome defects), mid piece (Proximal and distal droplets) and tail (broken tail, bent tail, double tailed, and coiled tail) abnormalities. At least 500 spermatozoa were counted in 5µl of the sample at 400x magnification.

Concentration: it was determined as billions/ml using calibrated spectro-photometry (IMV Technologies; France) as described by Rai *et al.* (2017). Procedurally; a blank tube was loaded with formol-buffered saline and inserted into the machine with the clear sides on the left and right for calibration. Then, the sperm suspension was prepared with 40µl of semen in 3960µl of 0.5M of NaCl in a 5ml cuvette and the absorbance reading was taken at 530nm. The machine measures the amount of light that passed through a sample and calculates the concentration of cells based on the density reflects. Print-out containing analysis date, sample number, ejaculate volume, extender volume, possible number of straws to be produced, total concentration and package concentration per straw were recovered from the machine.

Functional Tests: HOST and acrosome integrity tests were employed for the detection of plasma membrane and acrosomal integrity of spermatozoon in this study. HOST was carried out as per the method described in Zubair *et al.* (2013). Firstly; 0.735g of sodium citrate and 1.351g of fructose in 100 ml distilled water and 1.47g of sodium citrate and 2.702g of fructose in 100 ml distilled water were dissolved to produce the respective 150 mOsm/L hypo-osmotic solution and 300 mOsm/L control solution as described in Danilda *et al.* (2014), and both of the solutions were maintained at 37^oC for 5 minutes before use; then 1ml of each solution was mixed with 0.1ml frozen thawed semen in a test tube and incubated for 60 minutes at 37°C. Immediately after incubation, each sample was fixed with 0.1ml of 10% formaldehyde to retain the shape for subsequent

observation. A drop of well mixed semen sample (for the control and HOST) was placed on a glass slide and covered with cover slip. A total of 500 sperm cells were counted for non-reactive (sperm cells whose tails were straight) and reactive spermatozoa (sperm cells whose tails were swollen and/or curled) in at least 5 different fields of vision at 400X magnification for each of the control and HOST. HOST reactive spermatozoa percentage was determined by deducting the number of reactive spermatozoa in the control solution from the number in hypo-osmotic solution.

Acrosome integrity test was conducted for frozen thawed semen using Trypan blue-Giemsa dual stain as described in Boccia *et al.* (2016). Procedurally; the frozen semen was thawed in a water bath at 37°C for 30 s and diluted in 0.9% saline water at a dilution of 1:9 semen to saline ratio. After gentle mixing of one drop of diluted semen with one drop of 0.27% trypan blue, smear was made on a slide and air dried at room temperature. Then the smear was fixed in a solution of 86ml 1N HCl and 14ml 37% formaldehyde for two minutes and rinsed with water. The smear was again stained with 7.5% (v/v) giemsa overnight (16-20hrs). After washing and drying of the smears, samples were viewed at 1000x oil immersion to count at least for 200 spermatozoa. Based on the stain characteristics of the acrosome, results were classified as: acrosome intact live (AIL: when the whole sperm stains only with giemsa), acrosome intact dead (AID: when the arosmal region stains with giemsa while the tail and neck regions stains with trypan blue), acrosome lost live (ALL: when the arosme stains with trypan blue while the tail and neck regions stain with giemsa) and acrosome lost dead (ALD: when the whole sperm stains only with trypan blue).

Advanced Semen Evaluation: after the initial gross and microscopic evaluation, the semen was further subjected to a computer based integrated semen analysis system (ISAS® Prosier, Spain) with strict adherence to the program settings provided by the manufacturer for bovine semen. To perform the evaluation, aliquot of 100µl of fresh or chilled diluted semen was placed into a pre warmed micro-centrifuge tube and re-diluted at a rate of 1:3 (semen to extender) to bring the concentration of spermatozoa 20 - 50 millions/ml. Sperm kinetic parameters and motion characteristics were recorded (Table 3):

Table 3. Sperm kinetic parameters and motion characteristics of bull spermatozoa as measured by ISAS

Sperm kinematic	Description	Sperm motion characteristics
VCL (µm/s)	Curvilinear velocity	Slow
VAP (µm/s)	Average path velocity	Medium
VSL (µm/s)	Straight line velocity	Medium Progressive
ALH (µm)	Amplitude of lateral head displacement	Rapid
BCF (Hz)	Beat cross frequency	Rapid Progressive
STR (%)	Straightness	Progressive
LIN (%)	Linearity	Non progressive
WOB (%)	Wobble	

Additionally, total motility percentages (TMO, %) and progressively motile percentages (PROG, %) were also recorded.

3.3.3. Effect of risk factors on semen quality

To investigate the effect of general and reproductive health problems (IBR, BVD, TB and Brucellosis) on semen quality, all the study bulls were clinically examined and serum samples were harvested and stored at -20 degrees until submission and/or test. Portion of serum samples for the investigation of IBR and BVD from each study bull were submitted to the national animal health diagnostic investigation center (NAHDIC). Other portions of serum samples from each bull were tested for bovine brucellosis using rose bengal plate test (RBPT) at Livestock Development Institute. While tuberculosis was investigated using comparative intradermal tuberculin test (CITT) at Livestock Development Institute.

Bovine Tuberculosis: Investigation of bovine tuberculosis using CITT was conducted as it was described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial

Animals (2008/9). Procedurally, the test was applied in the middle third of the neck of each study animal by shaving two sites at around 12 cm apart. The thickness of the skin was measured with a caliper (Hauptner Herberholz, Germany) and avian-purified protein derivative; D4 ER strain (PPD-A) 25,000 IU/mL (Lot 140404) and bovine purified protein derivative AN5 strain (PPD-B) 30,000 IU/mL (Lot 906218A) from Prionics Lelystad B. V. (The Netherlands) were injected (i.e., 0.1 mL of PPD-B at one shaved area and 0.1 mL of PPD-A at the other shaved area). Skin thicknesses were again measured using caliper 72 hours after PPD injections. Interpretation of the result was based on the observation for skin fold measurement difference; viz. a bovine reactor was defined for the animal in which the relative increase in skin thickness at the injection site for PPD-B was at least 4 mm greater than the increase in skin thickness at the injection site for PDD-A. A negative reactor was identified when there is no reaction to the bovine antigen or when the difference of the skin thickness at the injection sites did not exceed 2 mm. An inconclusive reaction was recorded if reaction to both PPD-B and PPD-A exceeded 2 mm, but the difference between the bovine and avian reaction was < 4 mm. However, since all the study bulls were negative to CITT; retesting for conformation of inconclusive animals after 60 days was not needed and conducted.

Bovine Brucellosis: Rose bengal plate test for bovine brucellosis investigation was performed as per the method described in Chisi *et al.* (2017). Briefly; 10 ml of blood sample was collected from the jugular vein of each study bull using plain vacutainer tube and each sample was labeled using codes, describing the specific bull identification. The tubes were placed tilted on a table over night at a room temperature to allow clotting and by next morning, the clotted blood in the tubes was centrifuged at 3000g for 20 minute to obtain clear serum; and to perform the test, 30 μL of RBPT antigen and 30 μL of the test serum were placed alongside on the plate, and mixed thoroughly. Then the plate was shaken for 4 minute and the degree of agglutination reactions was recorded. The sample was classified as positive if any agglutination is observed and as negative if no agglutination. However, since all the study bulls were negative to the screening RBPT; conformation using complement fixation test (CFT*)* was not conducted for the study animals.

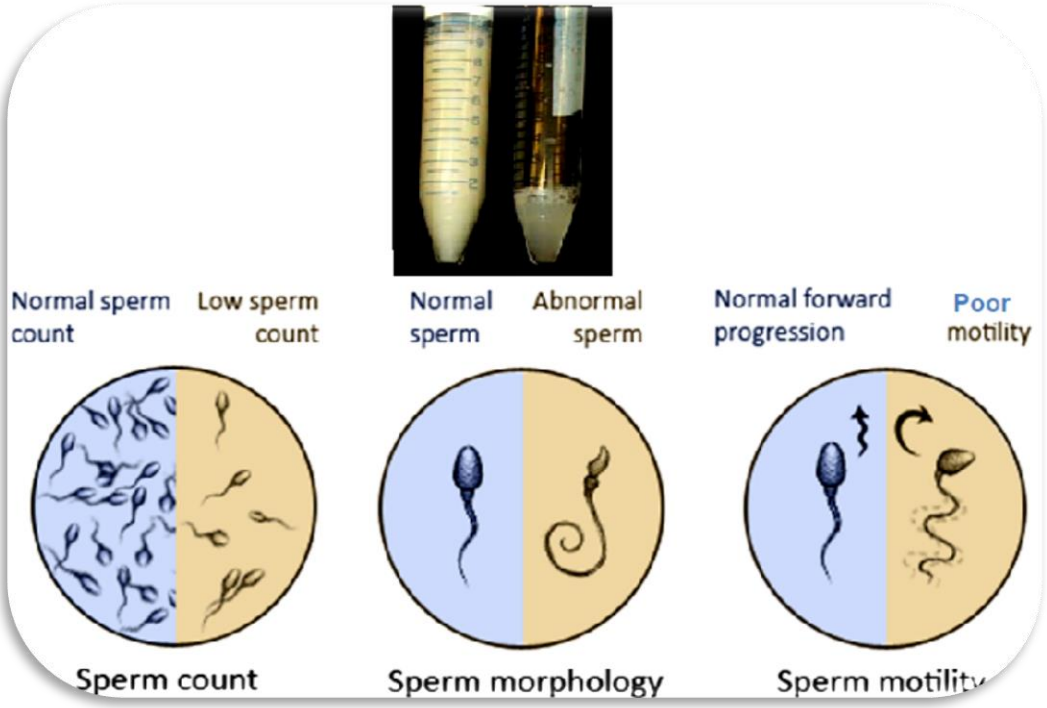
Bovine Viral Diarrhea: Detection of the antibody level specifically produced as a result of bovine viral diarrhea virus (BVDV) antigen in the serum was examined by indirect antibody enzyme-linked-immunosorbent-assay (I-ELISA) as it was described in Nigussie *et al.* (2010). However, all the bulls had been negative for the antibody specifically produced as a result of bovine viral diarrhea virus (BVDV) antigen.

Infectious Bovine Rhinotracheitis: similar to BVD, this was also detected using indirect antibody enzyme-linked-immunosorbent-assay (I-ELISA) for the presence of infectious bovine rhinotracheitis virus antigen; as it was described in Sibhat *et al.* (2018). However, in this case also all the bulls had been negative for the antibody specifically produced as a result of infectious bovine rhinotracheitis virus antigen.

3.4. Data Preparation and Statistical Analysis

The data obtained from each parameter were entered to Microsoft excel sheet (MS-Excel, 2010) and SPSS computer statistical package for windows (SPSS, 2016 Version 16.0, USA) was used for analysis. Semen quality was determined from values of functional evaluation test (plasma and acrosome membrane integrity), data populated from both the conventional and advanced (ISAS) semen evaluation parameters. Descriptive statistics and percentage were used for comparisons. Quantitative data: PH, volume, concentration, mass activity, individual motility, sperm morphological defects, acrosome integrity, plasma membrane integrity, live to dead spermatozoa ratio and kinematic parameters were compared using Analysis of Variances (ANOVA). Duncan's Multiple Range Test (DMRT) was used to compare breed differences, for those means and percentages at a probability level of 5%. Test of agreement between the conventional and advanced methods for spermatozoa motility evaluation was carried out for each breed at each stage of production to determine what proportion of the routine methods is invalidated by the advanced technique. Moreover, correlation among semen quality evaluation methods for pooled frozen semen data was computed using Pearson correlation (r). Chi square test was also used to make specific comparison between evaluation methods and for qualitative data among breed. In the analysis, $P < 0.05$ was set for level of significance.

Chapter IV



RESULTS

4.1 Breeding Soundness Evaluation

Table 4 illustrates how some of the evaluations were carried out in the bulls. In all the breeding bulls, testicular symmetry and movability in the scrotal sac and seminal vesicle development upon rectal palpation were optimal and acceptable for the requirements of these parameters as a breeding bull. However seminal vesicles were relatively smaller in Boran bulls.

Table 4. Subjective breeding soundness evaluation parametric values among breeds

BSE parameters	Breed type		
	Boran (n=4)	Cross (n=4)	HF (n=6)
Testicular symmetry	√	√	√
Testes movability in scrotal sac			
Right testis	√	√	√
Left testis	√	√	√
Seminal vesicle development			
Right Seminal vesicle	√	√	√
Left Seminal vesicle	√	√	√

Scrotal circumference was the smallest ($P < 0.05$) in Boran bulls (Table 5). No difference was observed in testicular measurements between Crosses and HF both based on age and body weight variations.

Table 5. Results (M ± SD) of breeding soundness evaluation among breeding bulls

Parameters	Breed type			P value
	Boran (N=4)	Cross (N=4)	HF (N=6)	
Scrotal circumference (cm)	33.2± 1.05[a]	37.1±2.72[b]	38.4±3.29[b]	0.03
Left testis				
Length (cm)	12.4±0.74	13.6±0.95	13.3±0.52	0.07
Width (cm)	6.5±0.33[a]	7.0±0.65[ab]	7.6±0.31[b]	0.01
Thickness (cm)	6.1±0.26	6.7±0.94	6.9±0.37	0.11
Volume (cm^3)	254.4±17.43[a]	341.6±104.75[ab]	367.5±41.08[b]	0.05
Weight (g)	264.1±18.1[a]	354.5±108.73[ab]	381.4±42.5[b]	0.05
Right testis				
Length (cm)	13.0±0.63	14.1±1.17	13.2±0.90	0.26
Width (cm)	6.6±.28[a]	7.1±.52[ab]	7.6±.52[b]	0.03
Thickness (cm)	6.4±.35	7.3±1.01	7.2±.51	0.14
Volume (cm^3)	287.1±18.80	388.4±113.67	378.0±49.71	0.11
Weight (g)	298.0±19.51	392.4±117.98	403.2±51.60	0.11
Libido (score)	2.94±.34	2.97±.17	3.00±.00	0.40
Body weight (kg)	459.3±42.29[a]	812.5±100.78[b]	851.7±135.67[b]	0.00
Age (months)	34.4±0.24[a]	63.5±16.09[b]	66.3±10.81[b]	0.01

Values across rows with different super scripts are significantly different at P<0.05.

4.2. Conventional Semen Evaluation

Semen volume, pH and sperm morphological defects were significantly different (*P<0.05,* Table 6) among the different breeds with the highest abnormality recorded in cross breed bulls (18.1%). Boran bulls produced comparatively smaller volume of semen. Spermatozoa head abnormality was significantly higher in Boran breed (*P<0.05*) while tail abnormality was highest both in Boran and HF bulls. Interestingly, proximal droplets was 8-16 times higher (*P<0.05*) in Cross breed bulls compared to the Boran and HF bulls.

There were no significant differences in concentration and both in mass activity and individual progressive motility among the three breeds.

Table 6. Measures of semen parameters (M ± SD) in fresh ejaculates using conventional method in Boran, HF and Cross breed bulls used for semen production at LDI

Semen quality measures	Breed type of the bulls			*P* value
	Boran (n=35)	Cross (n=33)	HF (n=57)	
Volume (ml)	9.81 ± 2.35^{a}	11.29 ± 2.55^{b}	11.39 ± 3.11^{b}	0.02
PH	6.31 ± 0.08^{a}	6.42 ± 0.12^{b}	6.58 ± 0.12^{c}	0.00
Concentration (Bil/ml)	1.03 ± 0.29	1.16 ± 0.28	1.16 ± 0.29	0.09
Mass activity (score)	3.29 ± 0.46	3.24 ± 0.44	3.35 ± 0.48	0.55
Morphological defects (%)				
Head	5.02 ± 3.97^{b}	4.66 ± 3.16^{ab}	3.42 ± 2.14^{a}	0.03
Mid piece	0.31 ± 0.57	0.35 ± 0.49	0.18 ± 0.36	0.18
Tail	4.70 ± 3.52^{b}	3.16 ± 2.30^{a}	4.91 ± 3.42^{b}	0.04
Proximal droplet	0.53 ± 0.10^{a}	8.63 ± 2.62^{b}	1.01 ± 0.21^{a}	0.00
Distal droplet	1.01 ± 0.21	1.30 ± 0.31	0.93 ± 0.21	0.55
Total abnormality	11.57 ± 1.05^{a}	18.10 ± 2.31^{b}	10.44 ± 0.63^{a}	0.00

Values across rows with different super scripts are significantly different (P < 0.05), n = number of ejaculates

4.3. Semen Functional Tests

Proportion of HOST reactive sperm in frozen semen was significantly (*P<0.05,* Table 7) different between Boran (39.55%) and HF breeds (49.46%). Alike to HOST, there was a significant (*P<0.05*) difference among breeds in the proportion of acrosome intact live spermatozoa (Table 7). As presented in the table, extra percent live spermatozoa were also recorded in the order of 22.23%, 18.06% and 17.72% for Boran, Cross and HF breeds, respectively; however the acrosomes of these percentages of spermatozoa for each breed were not intact. The lowest and highest HOST reactivity (39.5 and 49.5%) directly

corresponded (R = 0.88) with lowest and highest percent acrosome intact sperms (67.57 and 72.93%) in Boran and Holstein bulls, respectively.

4.3.2. Live:dead spermatozoa ratio

No significant difference was observed for live:dead spermatozoa ratio among the three breeds; with the highest ratio recorded corresponding to samples from HF bulls that also had the highest HOST reactivity.

Table 7. Semen quality for functional tests (M ± SD) among breeds for frozen semen

Traits of semen quality	Breed type			*P* value
	Boran (n=35)	Cross (n=33)	HF (n=57)	
HOST reactive (%)	39.55± 12.9[a]	45.24±14.04[ab]	49.46±16.94[b]	0.01
Live:Dead spermatozoa ratio	2.16±0.89	1.96±0.54	2.21±0.63	0.26
Acrosome integrity (%)				
Acrosome intact live	43.29±1.92[a]	46.9±1.75[ab]	49.67±1.30[b]	0.02
Acrosome intact dead	24.29±2.34	24.76±1.84	23.26±1.27	0.81
Acrosome lost live	22.23±2.15	18.06±2.00	17.72±1.32	0.15
Acrosome lost dead	10.20±0.93	10.27±.94	9.35±0.64	0.64
Total acrosome intact	67.57±2.98	71.67±2.78	72.93±1.81	0.27

Values across rows with different superscripts are significantly different (P<0.05), n = number of ejaculates

4.4. Advanced Semen Evaluation

All sperm kinematic parameters (VCL, VAP, VSL, ALH, BCF, LIN, STR, and WOB) were significantly (*P<0.05*) different for all stages of semen production among the three breeds. VAP, VSL, BCF, LIN, STR and WOB were significantly higher (P<0.05) in fresh samples of HF bred bulls compared to Boran and Crosses (Table 8). Conversely, VCL,

VAP, VSL, ALH, BCF, LIN, STR, and WOB were significantly ($P<0.05$) lower in all fresh, chilled and frozen samples from Cross bred bulls. However, in contrast to fresh and chilled semen, VCL, VAP, VSL, ALH, BCF, LIN, STR, and WOB were significantly ($P < 0.05$) higher in frozen semen of Boran bred bulls.

Table 8. Kinematic parameters (M ± SD) of spermatozoa among breeds at fresh, chilled and frozen stages of production

Semen type	Breed	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (HZ/s)
Fresh	Boran	234.5±98.4^{c}	129.2±60.9^{b}	73.3±52.5^{b}	32.4±31^{a}	55.2±54^{a}	55.7±54^{a}	5.0±.4.8^{c}	19.2±1.8^{b}
	Cross	183.2±94.5^{a}	102.6±57.0^{a}	58.2±44.6^{a}	34.1±33^{b}	56.2±54^{b}	57.1±55^{b}	4.1±.3.9^{a}	16.8±1.6^{a}
	HF	229.1±98.1^{b}	134.7±61.4^{c}	78.5±50.6^{c}	36.5±35^{c}	58.8±.57^{c}	59.4±58^{c}	4.8±.4.6^{b}	19.9±1.9^{c}
Chilled	Boran	210.7±106^{b}	108.4±53.6^{b}	61.4±41.7^{b}	31.5±18^{b}	56.6±26^{c}	53.5±16^{a}	4.9±2.3^{b}	15.9±.8.0^{b}
	Cross	194.0±107^{a}	100.9±56.9^{a}	52.0±39.6^{a}	29.9±20^{a}	52.4±27^{a}	53.7±17^{a}	4.5±2.3^{a}	15.4±8.1^{a}
	HF	216.2±103^{c}	118.5±59.0^{c}	62.5±43.3^{c}	31.3±19^{b}	54.0±26^{b}	55.7±15^{b}	4.9±2.2^{c}	16.7±8.8^{c}
Frozen	Boran	169.3±96.3^{c}	94.4±54.1^{c}	63.2±44.3^{c}	38.5±21^{c}	65.5±29^{c}	57.0±20^{c}	3.9±2.1^{c}	17.0±9.7^{c}
	Cross	145.7±92.0^{a}	81.1±51.9^{a}	49.5±40.3^{a}	35.4±22^{a}	59.9±29^{a}	56.7±21^{a}	3.4±2.0^{a}	15.4±8.9^{a}
	HF	153.7±83.7^{b}	86.3±49.5^{b}	55.9±40.2^{b}	36.6±19^{b}	62.5±27^{b}	56.7±18^{b}	3.6±1.7^{b}	16.4±8.9^{b}

Values for each semen type bearing different superscripts are statistically significant (P<0.05)

Summary of motion characteristics of spermatozoa tested at different stages of semen production is presented in table 9. Motion characteristics of spermatozoa (medium, and medium progressive) were significantly ($P<0.05$) higher in fresh semen of crosses compared to Boran and HF breeds. In contrast; rapid motion characteristic was significantly ($P<0.05$) lower in this bred bulls. On the other hand; though the total individual motility in fresh semen was significantly higher ($P < 0.05$) in Boran breed; medium and non-progressive motion types, and individual motility were significantly lower ($P<0.05$) for this bred bulls in chilled semen (Table 9 and 10). In this stage of production, the highest progressive and total motility were recorded in the semen of HF bred bulls. In line with chilled semen, significantly higher ($P < 0.05$) total motility percentage (42.9%) for frozen semen was also observed in HF breed. Moreover, significantly lower ($P < 0.05$) progressive and rapid progressive motion characteristics were recorded in Crosses.

Table 9. Influence of breed on motion characteristics (M ± SD) of bull spermatozoa at different stages of semen production

Semen type	Motion type (%)	Breeds			*P value*
		Boran	Cross	HF	
Fresh	Static	17.2±8.3	21.3±8.9	20.7±9.1	0.08
	Slow	2.3±1.8	2.8±1.8	2.3±1.4	0.31
	Medium	3.9±4.2[a]	9.1±6.4[b]	5.4±4.1[a]	0.00
	Medium progressive	3.2±3.6[a]	7.4±5.3[b]	4.6±4.7[a]	0.01
	Rapid	42.2±19[b]	32.6±18.6[a]	37.0±15.1[ab]	0.05
	Rapid progressive	31.1±15	26.8 ±14.7	30.0± 13.1	0.39
	Total progressive	34.3±16	34.2±14.6	34.6±12.9	0.99
chilled	Static	41.0± 16.9[b]	32.2±10.1[a]	29.1 ±14.5[a]	0.00
	Slow	3.4± 2.5	3.8±2.2	3.0 ±2.01.7	0.14
	Medium	5.8 ±3.7[a]	9.5±6.2[b]	7.7±4.6[ab]	0.01
	Medium progressive	3.8±4.0	5.1±4.0	3.6±3.4	0.16
	Rapid	30.4±17.2[a]	32.8±15.7[a]	37.3± 17.0[b]	0.05
	Rapid progressive	15.6±9.3	16.6 ±9.5	19.3 ±10.4	0.15
	Total progressive	19.3±10.7	21.7±11.4	22.9±10.7	0.28
Frozen	Static	64.0 ±14.4[b]	64.8 ±17.5[b]	55.5 ±17.2[a]	0.01
	Slow	4.2 ±1.9	5.0 ±2.0	4.4 ±2.5	0.34
	Medium	4.6 ±3.2[a]	7.6 ±5.1[b]	6.6 ±3.3[b]	0.01
	Medium progressive	3.3±2.9	3.9 ±2.9	4.5 ±3.0	0.20
	Rapid	10.7±8.7	10.0 ±8.8	13.1± 8.9	0.21
	Rapid progressive	13.1 ±7.5[b]	8.7 ±7.8[a]	15.9 ±9.8[b]	0.00
	Total progressive	16.4±7.4[a]	12.6±8.6[a]	20.4±9.4[b]	0.00

Values in each row bearing different superscripts for each production stage are statistically significant (P < 0.05).

Table 10. Individual motility and loss of motility (M ± SD) across semen production stages as evaluated using conventional method and ISAS among breeds of study bulls

Method of evaluation	Breed	Individual motility across production stages			Loss of motility across production stages		
		Fresh	Chilled	Frozen	Chilled	Frozen	Total
Conventional method	Boran	77.29 ± 4.43	73.71 ± 4.90^{a}	45 ± 4.20^{a}	3.57±4.8^{b}	28.71±5.9	32.29±11.7^{b}
	Cross	77.27 ± 4.35	74.7 ± 4.99^{a}	48.03 ± 5.98^{b}	2.58±4.3^{b}	26.67±7.0	29.24±16.9^{a}
	HF	77.46 ± 4.13	77.11 ± 4.42^{b}	48.42 ± 5.60^{b}	0.35±1.5^{a}	28.68±6.7	29.04±14.4^{a}
ISAS evaluation	Boran	82.5±8.0^{b}	57.9±15.9^{a}	33.9±9.3^{a}	22.77±15.5^{b}	24.23±16.3	46.99±4.8^{b}
(Total ind. Motility)	Cross	76.8±9.3^{a}	67±9.6^{b}	33.1±12.8^{a}	8.27±8.4^{a}	31.94±16.7	40.22±4.3^{ab}
	HF	78.9±8.8^{a}	70.2±13.6^{b}	42.9±15.3^{b}	7.87±9.7^{a}	26.87±16.1	34.74±1.6^{a}

Values of each method bearing different superscripts for each production stage are statistically significant ($P < 0.05$).

As presented in table 10, significance differences ($P < 0.05$) for individual motility were recorded among the breeds in all stages of production except fresh semen (under conventional evaluation method). Loss of motility percentage was significantly high ($P < 0.05$) for Boran bred bulls at time of chilling and was comparable to its loss at freezing stage. Likewise, under both conventional and ISAS evaluation methods, the total motility percentage loss throughout the production was also significantly high ($P < 0.05$) for this breed.

4.5. Relationship of Semen Evaluation Tests

As presented in table 11, significance differences ($P < 0.05$) were investigated for motility values between evaluation methods of conventional and advanced (ISAS) in all stages of production except in fresh semen of cross and HF breeds. Likewise, though it was low, positive association (r=0.26) was also observed between motility percentages evaluated by ISAS and the conventional methods (Figure 5). On the other hand, a very high positive associations of individual motility (evaluated by ISAS) with HOST reactivity (r=0.71) and with acrosome intact live spermatozoa percentage (r=0.31) in frozen pooled semen samples of all bulls were investigated (Figure 3 and 4). In this study, individual motility percentage, estimated by conventional method, was not correlated with HOST reactivity.

Table 11. Influence of method of evaluation in predicting individual motility (Mean ± SD) at fresh, chilled and frozen stages obtained by conventional and ISAS

Semen type	Methods of evaluation	Breed			Total
		Boran (n=35)	Cross (n=33)	HF (n=57)	(n=125)
Fresh	Conventional	77.29 ± 4.43	77.27 ± 4.35	77.46 ± 4.13	77.3 ±4,2
	ISAS	82.5±8.0	76.8±9.3	78.9±8.8	79.7 ±8.9
	P	*0.001*	*0.98*	*0.17*	*0.007*
Chilled	Conventional	73.71 ± 4.90	74.7 ± 4.99	77.11 ± 4.42	75.5±4.9
	ISAS	57.9±15.9	67±9.6	70.2±13.6	67.1±14
	P	*0.000*	*0.004*	*0.001*	*0.000*
Frozen	Conventional	45 ± 4.20	48.03 ± 5.98	48.42 ± 5.60	47.3±5.5
	ISAS	33.9±9.3	33.1±12.8	42.9±15.3	39.0±13
	P	*0.000*	*0.000*	*0.033*	*0.000*

Values of each breed for each semen type are statistically significant (P<0.05) among the methods of evaluation, n = number of ejaculates

In all the breeds at chilled and frozen stages of production; the conventional method of evaluation significantly (*P < 0.05*) overestimates the motility percentage (Table 11). As presented in table 12, with minimum total motility cut-off values (40%) in frozen semen, nearly 56% of semen failed to fulfill the minimum approval requirements by ISAS.

Table 12. Proportion of semen doses approved by conventional Vs ISAS at a minimum individual motility cut-off value (40%)

Method of evaluation	Minimum cut-off values (40%)		X^2	*P* value
	<40	≥40		
Conventional (n=125)	-	125 (100%)	97.2	0.000
ISAS (n=125)	70 (56%)	55 (44%)		
Total (n=250)	70 (28%)	180 (72%)		

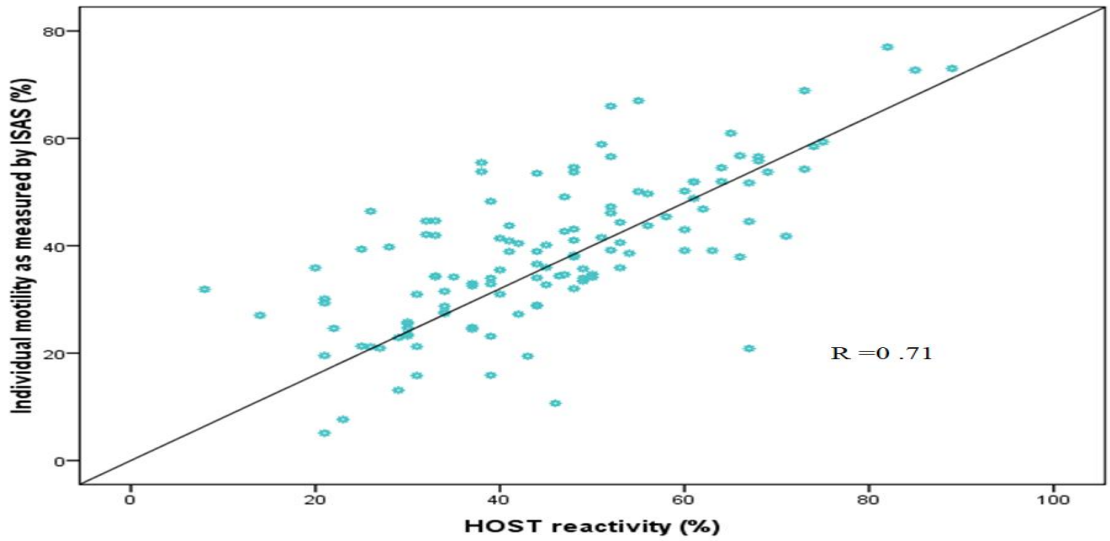


Figure 3. Correlation between individual motility (evaluated by ISAS) and percentage of HOST reactive spermatozoa in frozen samples of study bulls

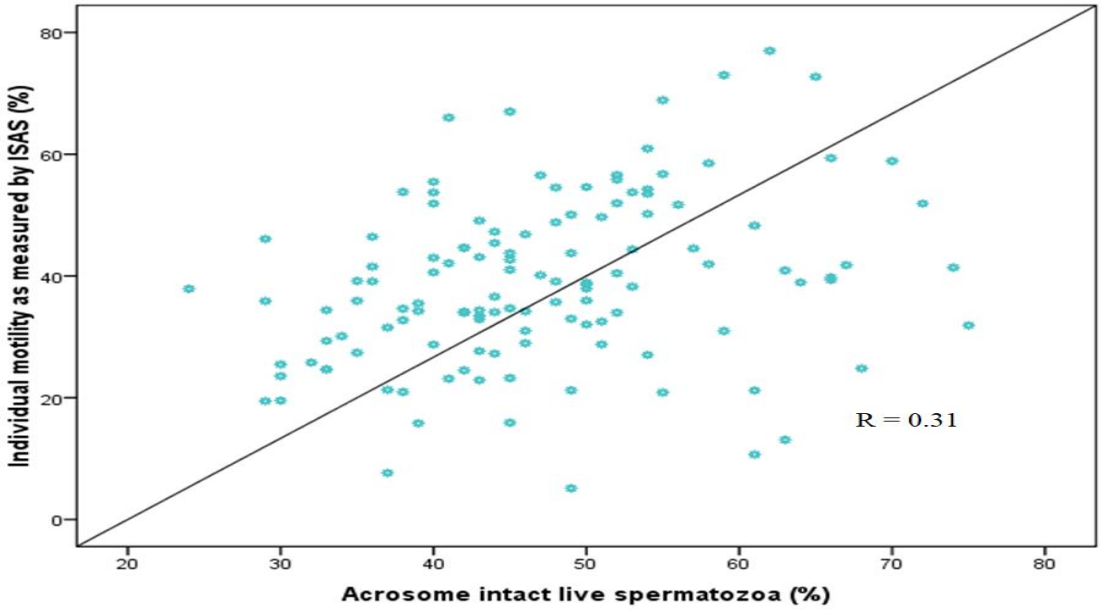


Figure 4. Correlation between individual motility (evaluated by ISAS) and acrosome integrity for live spermatozoa in frozen samples of study bulls

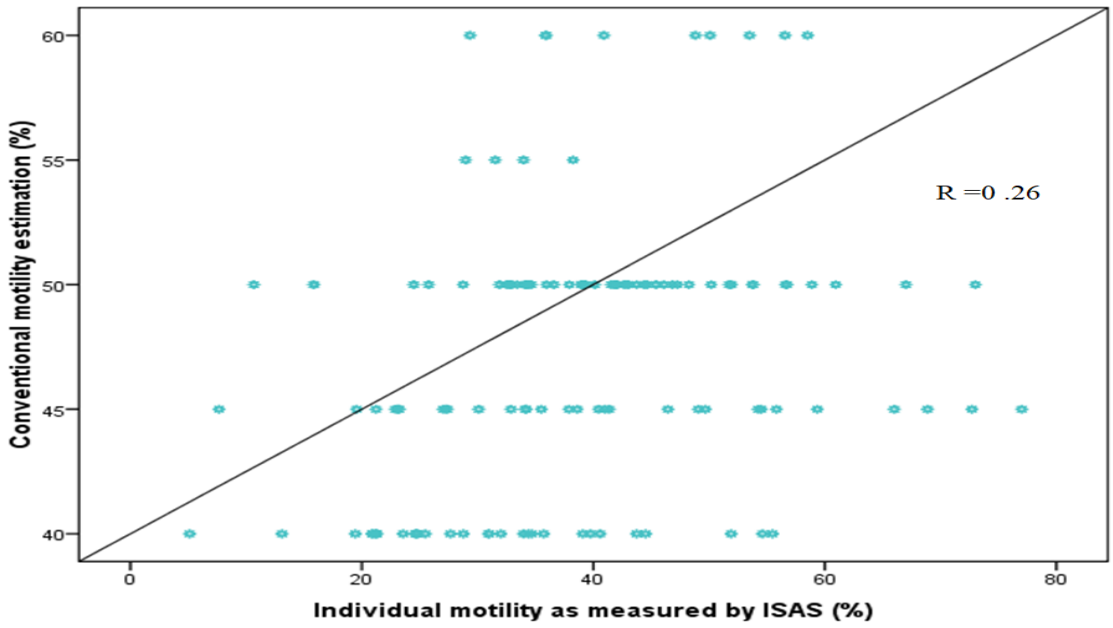


Figure 5. Correlation between the conventional and ISAS methods in estimating individual motility in frozen samples of study bulls

4.6. Effect of general and reproductive health problems on semen quality

All the bulls and/or breeds (Boran, Cross and HF) considered in this study were negative to the presumed diseases that potentially compromise the semen quality: Bovine tuberculosis, Infectious bovine rhinotracheitis, Bovine viral diarrhea and Bovine brucellosis (Table 13). All the semen parameters and motion characteristics of spermatozoa were derived from diseases free breeding bulls without the influence of the infectious diseases.

Table 13. Incidence of diseases among bull breeds

Disease tested	Breed type				X^2	*P* value
	Boran (n = 4)	Cross (n = 4)	HF (n = 6)	Total (n = 14)		
Bovine viral diarrhea (BVD)	-	-	-	-		
Bovine tuberculosis	-	-	-	-		
Bovine brucellosis	-	-	-	-		
Infectious bovine rhinotracheitis (IBR)	-	-	-	-		

Chapter V

DISCUSSION

The breeding soundness evaluations (testicular symmetry and movability in the scrotal sac and seminal vesicle development) were within the acceptable range with no significant breed influence. Libido is one of the important breeding soundness evaluation parameter that determines the quality of semen. Bulls with poor libido are usually reluctant to mount, and often gives semen that is small in volume and poor in quality (Rehman *et al.,* 2016). HF dairy bulls exhibit good libido with better semen quality (Ahmad *et al*., 2005); this also agrees to the finding in this study. Scrotal circumference is also influenced by breed as well as age of the bull. The sizes recorded in Boran bulls were in agreement with previous report by Lemma and Shemsu (2015) for pre-service young bulls in the same AI center. Study of Beggs (2013) indicated that normal spermiogram was most likely associated with scrotal measurements between 31 and 44 cm which in turn associated with good quality semen. All scrotal measurements in the present study fall within the range previously described for breeding bulls. Scrotal circumference and its heat regulation mechanism increases linearly with age until the rate at which breakdown of testicular tissues become faster than replacement (Brito *et al.,* 2002). This is believed to be one of the factors for the variation in semen quality among breeds and/or bulls. Moreover, fat deposition in the scrotum which is linearly associated with scrotal circumference also contribute to the variation in efficiency of scrotal thermo-regulation which in turn has impact on semen quality (Fair and Lonergan, 2018).

The presence of breed difference on semen quality (pH, volume, concentration, morphological abnormalities and motility of spermatozoa) has also been previously reported (Brito *et al.,* 2002; Andre *et al.,* 2017; Sitali *et al*., 2017). Moreover, values of the semen parameters were consistently different at different stages of the semen production (chilling and freezing). Some breeds of bulls such as HF breeds are known for production of larger volume of ejaculate compared to the indigenous Boran. Boran bulls had high percentages of head and tail abnormalities in fresh semen and low individual motility in chilled semen. The Borans used in the present study are unimproved breeds that have not been selected for any trait. On top of this, breed differences on several semen quality parameters have been previously reported by different authors (Blezinger, 1999; Andrabie *et al.,* 2002; Brito *et al.,* 2002; Andre *et al.*, 2017; Sitali *et al*., 2017). It is

very important to note that fertility from such bulls cannot be reliable as it is also evidenced by the poor results of the semen evaluation.

Breed specific differences in semen characteristics have been previously reported (Sori, 2004; Sinishaw, 2005; Gebre-Medihin *et al.*, 2007; Demeke, 2010; Lemma, 2011; Lemma and Shemsu, 2015). Semen quality from Boran bulls rapidly deteriorated after chilling, as shown by the higher rate of reduction in individual spermatozoa motility concomitant with the period of semen equilibration both during conventional and ISAS evaluation. This might give a clue as to what extent should the duration of semen equilibration be extended for this particular breed. In previous experimentation with in vivo embryo production using Boran semen showed a very low fertility resulting in a higher proportion of unfertilized ovum (Degefa *et al.,* 2016) which might be associated with the process of freezing, influencing the semen fertilizing ability. Optimization of the chilling and freezing protocol for this specific breed becomes warranted to improve conception rate when the semen is particularly destined for use in AI. HF breeds are known to produce relatively good quality semen (Bang, 2008). This was also evident in this study during the evaluation of the semen by the conventional method. However, Engidawork (2018) did not confirm difference of semen quality among breeds; which might be due to his smaller number of observations in the study. Further, difference in age, nutritional status, season of the year, method of semen collection procedure and frequency of collection also result in some form of variation in semen quality (Blezinger, 1999; Andrabie *et al.,* 2002). For instance, Blasco (1984) and Farrell *et al.* (1995) indicated that factors like degree of sperm maturation, stores (ATP), presence of surface-active agents in the cell membrane (agglutinins and detergents), viscosity of the fluids, osmolarity and ionic composition of seminal plasma can also affect semen quality and bring variation among breeds.

According to World Health Organization (2010) recommendation; when percentage of non-motile spermatozoa exceeds 40%, it is clinically important to verify the spermatozoa live:dead ratio of that sample. At the LDI, where this study was conducted, post freezing minimum threshold for motility is 40%; implicating high chance of getting more than

40% non-motile spermatozoa; and this situation enforces the laboratory to assess the proportion of live spermatozoa for the semen produced at the institute. Moreover, accurate motility evaluation can also be verified by sperm vitality tests, as the percentage of dead spermatozoa would not be higher than the percentage of non-motile spermatozoa (Chemes and Rawe, 2003). In this study the percentages of non-viable spermatozoa were less than 40% for each of the three breeds although a relatively higher live:dead spermatozoa ratio was recorded in HF breed; which also again implicates and supports the evidence described by Bang (2008) for HF semen.

An intact and functionally active spermatozoa membrane is required for cell metabolism, capacitation, acrosome reaction, attachment and penetration of the oocyte (Dhurvey *et al.*, 2012). Thus, assessment of the sperm membrane functional status appears to be a significant marker for the fertilizing capacity of spermatozoa (Zubair *et al.,* 2013). The poor HOST reactivity in Boran breed could be due to the high incidence of tail abnormality for this breed. Breed differences has also been previously reported in Red Sindhi, Crossbred, Haryana and Jersey breeds (Mishra *et al.,* 2013) with an increasing of the incident with high ambient temperature. The mean HOST reactive percentages of spermatozoa reported for Sahiwal, (47%) and HF (40%) semen (Zubair *et al*., 2013; Zodinsanga *et al.* (2015) are closely similar to the present finding. On the other hand, Zodinsanga *et al.* (2015) reported HOST reactive spermatozoa percentages of 32.6 for pooled frozen semen of pure breeds (HF, Jersy and Sahiwal) and 48.4 for their Crosses pooled frozen semen also showing the presence of breed differences. Vincent *et al.* (2012) recommended a cutoff value (40%) for membrane intact cells in bovine frozen semen.

Seasonal variation in HOST reactivity of spermatozoa has been discussed in some breed (Nordin *et al*. (1990). Management differences related to sheltering has resulted in highest HOST reactivity in buffalo during summer (Koonjaenak *et al.,* 2005). Anecdotal information indicates that field performance of Boran semen is very low as evidenced by the low conception rate (Degefa *et al.,* 2016). Application of a simple screening HOS Test, that seemed to have been related to sperm tail defect in Boran, is very useful in the

production of better quality semen for AI. HOST has proved to be most appropriate for predicting the fertilizing capacity of frozen-thawed than fresh semen (Colenbrander and Kemp, 2003) because membrane damage in frozen-thawed semen is a more important limiting factor than in fresh semen. Moreover, as spermatozoa with higher membrane cholesterol content is expected to resist destabilization of membrane following cryopreservation, this spermatozoa HOST reactive percentage difference among the study breeds might also attribute to varying concentration of their membrane cholesterol (Srivastava *et al*., 2013) which needs further investigation.

Interestingly, HOST values and acrosome integrity percentages did not show a clear association with motility parameters when semen was evaluated by the conventional method. However, a significant association was apparent when semen was evaluated using ISAS. This agreement confirms the superiority of the ISAS system over the conventional method in evaluating subtle sperm defects such as membrane and acrosomal defects that potentially influence fertility. Sperm rated to have a high motility value might not necessarily have higher fertilization potential, hence previous routine at LDI that employ only conventional method of analysis for semen destined for AI invariably implicate a poor performance at field level. Added advantage of the ISAS is its ability to identify sperm morphological defects that are highly correlated with individual motility. Spermatozoa with cytoplasmic droplet both at the proximal or distal end, though can be rates as motile during the conventional method, would be identified unfit for AI because of poor fertilization ability. Moreover, as motility is correlated with the integrity of the plasma membrane (Brito *et al.,* 2002); the ISAS system has the ability to filters and omits those sperm cells with poor kinematic parameters (such as VCL <10µm/s) and considers them as static cells (Holt *et al.,* 2007). Similarly, in some earlier studies, association has been established among semen quality parameters of sperm motility, viability, acrosome integrity and HOST reactivity (Kirk *et al*., 2005; Lodhi *et al*., 2008; Kumar, 2015).

The percentages of total acrosome integrity for each of the three breeds in this study were in agreement with the findings of Chowdhury *et al.* (2014) who also reported an overall acrosomal integrity of 73.74%, for Jersey, Cross-bred of Jersey, Gir and Murrah. Vincent

et al. (2012) also recommended a cutoff value (66%) for intact acrosome in bovine frozen semen. Similarly, Kumar *et al*. (2015) also reported acrosome intact percentages of 71.94 and 69.38 for frozen semen of Jersey and Jersey-cross bulls which were in close agreement with this study finding. Spermatozoa from HF breeds is known to have relatively high content of specific lipoprotein complex and enzymes (hyaluronidase, acrosin, esterases, acid hydrolases) and other proteinases which are known to be involved in the fertilization process (Garner, 1994). Fertility is affected by the structurally and biochemical integrity of the acrosome which contains such enzymes necessary for penetration through the outer layers of the ovum and achieve fertilization (Thundathil *et al.*, 2000).

The present study showed a significantly higher individual spermatozoa motility percentage (82.50%) in fresh Boran semen compared to Cross and HF breeds. However, this value drastically dropped during equilibration indicating the lack of tolerance for chilling temperature in this breed. As it was stated by Davis and Katz (1992) the conventional spermatozoa motility evaluations are not fully reliable to predict the fertility potential of bull semen. Hence, higher rating of motility parameters in fresh samples have to be re-evaluated at later stage using an advanced technique such as ISAS. Sperm motility is a readily identifiable test and is the most important feature of fertile spermatozoa that reflects several structural and functional competences.

Comparable percent individual motilities of 79.23%, 78.69% and 76.12% using the conventional method has been previously reported by Lemma and Shemsu (2015) in the same center for Boran, HF and Boran*HF Crosses, respectively. Galmessa *et al*. (2014) reported individual motility of 78.49% for fresh semen of Sahiwal breed using CASA system. This was very low (65.22%) in an earlier study by Keshav (1996) for the same Sahiwal breed. On the other hand, very high motility percentage of 94.3% was also reported for Jersey bulls (Sundararaman *et al*. 2012).

Breed differences in motility has been reported previously (Seyoum *et al*, 2021), which may also come as a result of high peroxidation of fatty acids that can leads to the loss of

membrane integrity of the spermatozoa, and which in turn affect the motility (Garner, 1994). It was confirmed that low HOST reactivity, a sign of poor membrane integrity, was evident in Boran breed. The fact that motility is highly rated in fresh samples and rapidly deteriorating in cooled ejaculate typically confirms the need for optimization of the cooling process for this breed. Further, the added toxic effect of glycerol on spermatozoa should also be separately investigated for Boran semen as this can also seriously affect sperm motility (Kadirvel *et al.,* 2009; Muhammmad *et al*., 2013). Furthermore, the release of phospholipids that is important for production of reactive oxygen species (ROS) which in turn is toxic for the normal spermatozoa might be high at this stage of semen production. In any case, motility below 50% has been known to be associated with low conception rate or poor fertility (David (2003). Chilled Boran semen at 57.90% motility found in this study is obviously marginal in performance.

As a result of accumulated cellular injuries that arise throughout the process (chilling and freezing), the lowest individual spermatozoa motility percentages were seen in Boran and Cross breeds after freezing and these motility percentages were in agreement with the study finding of Amanda (2011). The ISAS motility results for HF in this study was comparable to the study conducted by Lenz *et al*. (2010). The total and progressive individual motility percentages of frozen semen evaluated by ISAS in this study also falls within the ranges reported by Keshav (1996). Sundararaman *et al*. (2012) also reported a higher motility percentage for chilled (89.4%) and frozen (63.0%) semen in Jersey bulls. According to Alapati *et al.* (2009), for frozen semen to be considered as fertile at field conditions, its total motility must be above 33% under CASA system though older reports (Galli et al. 1991) indicate 22% as the smallest margin. Other researchers (Vincent *et al.,* 2012) recommended cut-off values 40% and 15% for total and progressive individual spermatozoa motility of frozen-thawed bovine semen evaluated with CASA to determine pass/fail rates during quality control immediately after thawing. Our finding in all study bulls (>33%) basically agrees with the former recommendation for total motility in all the breeds and also with Vincent *et al.* (2012) result for progressive motility of Boran and HF breeds when semen is evaluated by ISAS. However, with minimum cut-off value (40%) set by Vincent *et al.* (2012) and the LDI laboratory by itself for frozen semen under

conventional method of evaluation, nearly 56% of semen produced at LDI failed to fulfill the minimum approval requirements by ISAS. This was very apparent in semen of Cross for both total and progressive motility (33.1 Vs 12.9%) and to that of total motility (33.9%) in Boran semen. Nevertheless, LDI packs 30 million spermatozoa per straw for a single dose; this might compensate the fertility problem that could come from such type. Variations between studies might come from the differences in the minimum settings of the system, concentration of spermatozoa analyzed, counting-chamber depth, frame rates used for analysis, temperature at which semen was analyzed, type of extender used for dilution, and digitization threshold (Mortimer, 1994; Kraemer *et al.*, 1998).

Values of sperm kinematics in this study bulls can broadly be described as relatively lower in performance though the ALH, BCF, LIN, STR and WOB were comparable to reports by Sundararaman *et al.*, (2012). Further, the presence of breed differences in sperm kinematic values (VCL, VAP, VSL, ALH, BCF, LIN, STR, and WOB) has been confirmed in the current study that was even more evident in Cross breed bulls. On the other hand, except for VCL, VAP and VSL, the other values of the sperm kinematics in the present findings were comparatively higher than previously reported by Amanda (2011) and Galmessa *et al*. (2014) at all stages of production. Values for the ALH and BCF were also comparable to the findings by Waterhouse *et al.* (2010). A number of studies (Farrell *et al.*, 1995; Januskauskas *et al*., 1999; Gillan *et al*., 2005; Kathiravan *et al*., 2008) showed the presence of positive correlation between STR velocity of spermatozoa and field fertility. Madhuri (2012) also described a positive association of sperm kinematic values with cervical mucus and oocytes sperm penetration ability and with the results of in-vitro fertilization.

The relatively small kinematic values (VSL, LIN, STR and WOB) directly relates to the poor field performance of semen produced at LDI. The ISAS evaluation has also revealed a possible overrating by the conventional method since this was the routine practice. Even though microscopic sperm motility testing is the most commonly used parameter to determine the quality of semen intended for AI; this method is completely subjective and depends on the experience and ability of the person performing the test. It is no wonder

that at times a very low conception rate is reported at the field level (Hansar *et al* 2014; Ali *et al*, 2015; Jemal *et al* 2016, Degefa *et al.*, 2016) from semen produced at the former NAIC.

In contrast to motility percentages, the sperm kinematic values for post-thawed semen were significantly higher in Boran than the HF or cross breed bulls. This indicates that, sperms surviving the chilling process still have the chance to perform better after freezing particularly in Boran bulls; further confirming again the need for optimization of the cooling process. It is also worth noting that sperm kinematic values do vary for different reasons such as temperature at which the semen was analyzed, concentration, type of extender, digitization threshold, sampling technique, method of processing, time elapse between sampling and analysis, the accuracy of the specimen chambers used and the number of fields and spermatozoa examined (Verstegen *et al.*, 2002). The slow and medium spermatozoa motion characteristics were more apparent at all stages of the semen production in semen from cross breed bulls conversely influencing the proportion of medium progressive, rapid and rapid progressive spermatozoa in the total figure. On the other hand the overall progressive and rapid progressive motile spermatozoa percentages were higher for HF breed at all stages of the semen production.

Though clinical and laboratory disease testing for Bovine tuberculosis, Infectious bovine rhinotracheitis, Bovine viral diarrhea and Bovine brucellosis were conducted to see the effects of general and reproductive health problems on semen quality; all the bulls and/or breeds were found to be negative to all the diseases considered in the study. For this reason inferring their effects on semen quality could not be addressed by this study.

Chapter VI

CONCLUSION AND RECOMMENDATION

Breeding soundness evaluation (size of testis, scrotal circumference and libido) and semen quality parameters (semen volume, mass activity, individual progressive motility, concentration, pH and morphological defects) were generally within the range of previous reports. In this study, comparatively, HF bulls performed better in all respect compared to Boran and Cross breed bulls. As originally this breed was selected for dairy, better performance from HF bulls might be anticipated.

The multiparametric (ISAS) semen evaluation approach revealed the presence of breed differences in semen quality apparent from differences in individual progressive motility, sperm kinematics and motion characteristics at different stages (fresh, chilled and frozen) of semen production. This study confirmed that not all subtle differences in semen parameters that determine the quality of the semen produced for AI purpose were discoverable by the conventional method. Almost all sperm kinematic values and motion characteristics in the cross breed bull ejaculate were very low. Although semen parameters were known to be acceptable in fresh ejaculates from Boran bulls, the chilling process during semen equilibration dramatically reduced most of the motility parameters; further freezing process, though, did not seem to affect post thaw sperm kinematic values as revealed by the more stringent ISAS evaluation technique. This is a major area of concern for this breed semen, as the conventional evaluation method approves a semen which otherwise should have been rejected if LDI employed the ISAS system even at the start of the screening.

Individual progressive motility was comparatively low in frozen semen samples of Boran and cross bred bulls especially when evaluated using ISAS. The presence of very good correlation between the more simple functional tests and ISAS evaluation of sperm motility/kinematics that are useful in determining fertility is indeed very useful tool that can be exploited to implement when logistics do not allow for the use of the advanced method.

Kinematic parameters (VCL, VAP, VSL, ALH, BCF, LIN, STR, and WOB) were significantly different in all stages of semen production among the three breeds with the

lowest value recorded in crosses compared to either Boran or HF. These parameters were also known to have linear association with progressive motility estimated by the ISAS method. Unfortunately, the conventional spermatozoa motility evaluation in this study did not show significant association with values of sperm kinematics or motion characteristics evaluated by ISAS. Even, those associations that were detected in some of the semen parameters were not reliable. For instance, percentage of morphological defects showed a positive correlation with percentage of individual motility under the conventional semen evaluation method. However, this was not consistent with the ISAS values for percentages of motility. Hence, it seems that unless it is supported with other evaluation methods, the conventional spermatozoa motility evaluation alone was not enough and able to discover the high incidence of proximal droplet (as observed in cross breed bulls) which affects individual progressive motility.

Results of HOST reactivity and percentage of acrosome intact live spermatozoa were significantly different between Boran and HF breeds. Functional tests may not provide all the information about semen quality when they are conducted independently; but, they have the ability to predict the fertility potential of the semen when they are conducted together with other tests. Therefore, it can be safely concluded that HOST reactivity, percent acrosomal integrity and percent morphological defects in sperm cells are suitable test methods to grade semen quality especially when they are conducted synchronously with the ISAS method of semen evaluation.

Undertaking a multiparametric semen quality assessment using ISAS addressing various structural and functional aspects of the spermatozoa (motility, kinematic, motion characteristics and morphology) invariably improves the level of prediction to fertility and potentially screens the fertile and sub-fertile bulls in the semen production centers. Based on the above conclusive summary the followings are recommended

- Boran breed, that has recently received interest for its dual merit (beef and dairy) in Ethiopia, seems to require an optimization experiment in the semen freezing process. So far, we know that the equilibration of semen right before freezing (the

temperature where the semen pass from 37 ^{0}C to 4 ^{0}C) is the major area where motility was found to dramatically reduce

- Employing conventional semen evaluation method was not able to discover some the subtle defects which have huge effects on fertility of the breeding bulls, so introducing advanced multiparametric approach of semen evaluation addressing the structural and functional aspects of the spermatozoa is mandatory. These includes the plasma membrane and acrosomal integrity tests that are often affected by the freezing process
- Although breed difference on semen quality is vaguely described in many previous studies, it was also observed in this study, the underlying factors associated with each breeding bull, particularly the indigenous breeds have to be searched
- All bulls in the present study were free from diseases affecting the quality of semen. This is because of the stringent breeding soundness evaluation employed at LDI. Hence this must be maintained and further scaled up for other diseases as well as disease causing agents that could potentially survive the semen storage condition. And a specialized laboratory investigating semen contaminants should be set up

Chapter VII

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Chapter VIII

ANNEXES

Anex 1. Testicles, scrotal circumference and accessary sex glands development evaluation form

S.No.	Bull ID.	Testicular/ scrotal parameters															Accessory sex glands development (developed or not developed)						Remark	
		Size in cm											Symmetry		Mov.ity in scrotum									
		circumference	length		width		Thickness		Volume		Density	weight			Symmetry	Asymmetry	Movable	Unmovable	Seminal vesicle		Prostate		Cowers gl tos		Age in months	Body weight in Kg
			L	R	L	R	L	R	L	R		L	R						Dev	Und	Dev	Und	Dev	Und		
1.											1.038															
2.											1.038															
3.											1.038															
4.											1.038															
5.											1.038															
6.											1.038															
7.											1.038															
8.											1.038															
9.											1.038															

Anex 2. Data collection Form for Color, Volume, pH and presence of contaminants in semen

S.No	Bull ID.	Color of the semen					presence of contaminants				Volume in ml	PH
		creamy(5)	White(4)	Yellow(3)	watery(2)	Blood (4)	puss (4)	Blood (3)	Hair(2)	other debris(1)		
1.												
2.												
3.												
4.												
5.												
6.												
7.												
8.												
9.												
10.												

Anex 3. Data collection Form for Morphological status, mass motility, individual sperm cell motility, sperm cell concentration and Live:dead sperm count.

S.No.	Bull ID.	Morphological status for a total of 500 spermatozoa counted						Mass activity(0-4)	Indi. Motility (%)			Concentration (milli per ml)	for a total of 200 sperm cells			Remark
		Normal	Head abnormality	Mid-piece abnormality	Tail abnormality	prox. Droplet	Dist. Droplet		Fresh	chilled	Frozen		Live counts	Dead counts	Live:dead sperm ratio	
1.																
2.																
3.																
4.																
5.																
6.																
7.																
8.																
9.																
10.																

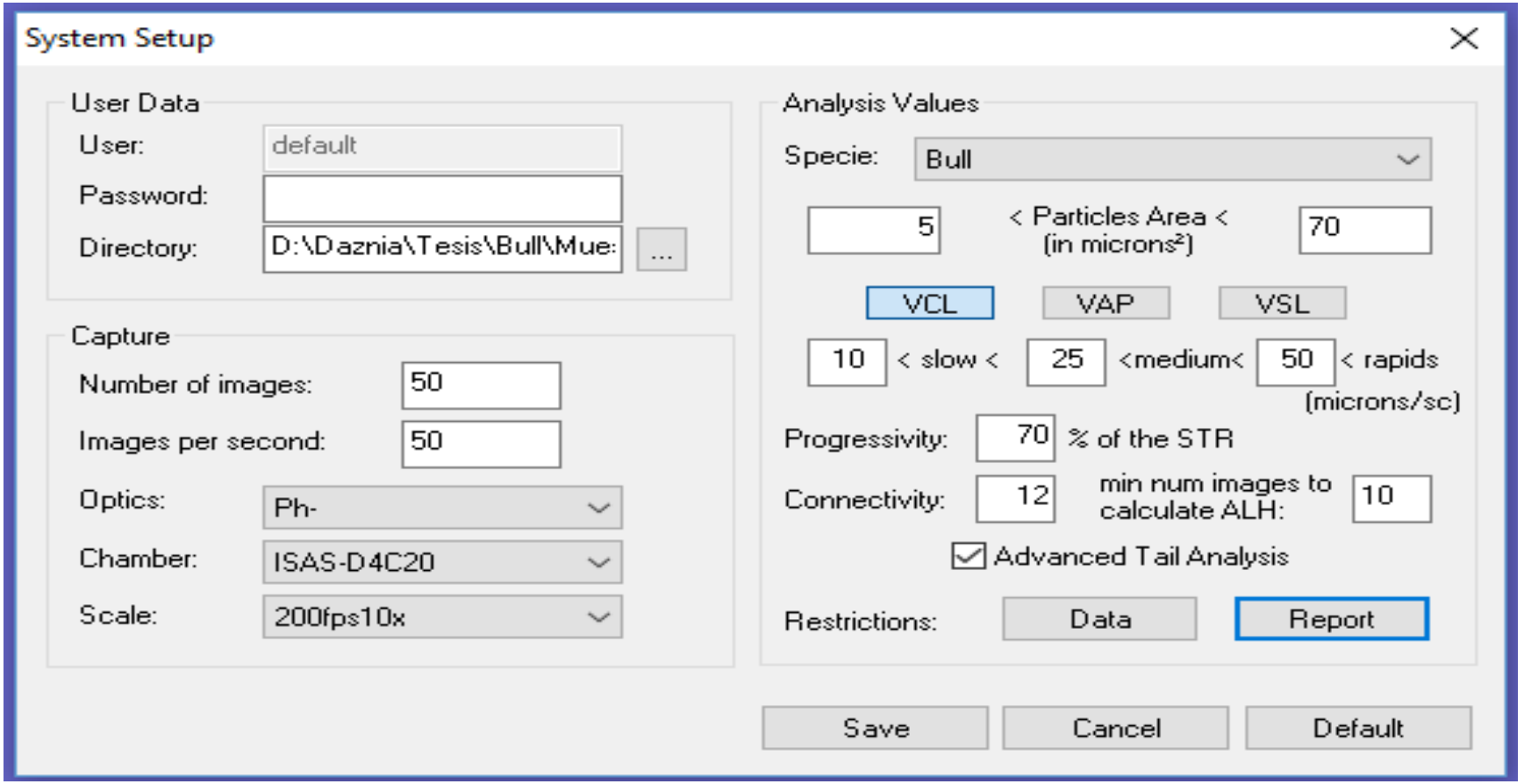
Anex 4. Data collection Form for Hypo-osmotic swelling test

S.No.	Bull ID.	Sperm tail status per 500 cells count in PBS		Sperm tail status per 500 cells count in hypo-osmotic solution		Deducted resultant /HOS-reactive spermatozoa/	Remark
		Normal tailed	Coiled tailed	Normal tailed	Coiled tailed		
1.							
2.							
3.							
4.							
5.							
6.							
7.							

Anex 5. Data collection Form for Acrosome integrity

S.No.	Bull ID	Acrosome integrity status for 200 cells count				Remark
		AIL	AID	ALL	ALD	
1.						
2.						
3.						
4.						
5.						
6.						
7.						
8.						
9.						
10.						
11.						
12.						
13.						
14.						

Anex 6. ISAS settings for bovine semen evaluation



Anex 7. Clinical and laboratory works of the study