ADDIS ABABA UNIVERSITY COLLEGE OF
VETERINARY MEDICINE AND
AGRICULTURE

COMPARISION OF CONCEPTION RATE OF FROZEN SEMEN FROM TWO EXTENDERS AND METHODS OF EARLY PREGNANCY DIAGNOSIS USING ULTRASONOGRAPHY, AND PROGESTERONE ANALYSIS IN DAIRY CATTLE

MVSc Thesis

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Comparison of Conception Rate of Frozen Semen from Two Extenders and Methods of Early Pregnancy Diagnosis Using Ultrasonography, And Progesterone Analysis in Dairy Cattle

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APPROVAL SHEET

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As member of the Examining Board of the final MVSc open defense examination, we certify that we have read and evaluated the Thesis prepared by: Abiy Shimelis, Title: **COMPARISION OF CONCEPTION RATE OF FROZEN SEMEN FROM TWO EXTENDERS AND METHODS OF EARLY PREGNANCY DIAGNOSIS USING ULTRASONOGRAPHY, AND PROGESTERONE ANALYSIS IN DAIRY CATTLE** and we recommended that accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Obstetrics and Gynecology.

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DEDICATION

This manuscript is dedicated to my Mama Selamawit Shewangizaw, my family and friends for nursing me with affection and love and for their dedicated partnership in the success of my life.

STATEMENT OF AUTHOR

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

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
BCS	Body Condition Score
CL	Corpus luteum
CR	Conception rate
DZARC	Debrezeit Agricultural Research Center
EIAR	Ethiopian Institute of agricultural research
EPF	Early pregnancy factor
FAO	Food and Agriculture Organization
FSH	Follicle stimulating hormone
FTAI	Fixed time artificial insemination
GnRH	Gonadotrophine releasing hormone
LH	Luteinizing Hormone
MoA	Ministry of Agriculture
MOET	Multiple Ovulation Embryo Transfer
NAIC	National Artificial Insemination Center
P4	Progesterone
PD	Pregnancy Diagnosis
PGF2α	Prostaglandin F2α
PR	Pregnancy rate
RMPT	Rapid milk progesterone test
RP	Rectal Palpation
TAI	Timed Artificial Insemination
US	Ultrasound

ABSTRACT

Semen extenders used in Ethiopia are imported and expensive. Pregnancy detection is mostly through rectal palpation. This study was designed to compare conception rates of frozen semen prepared by commercial (Bioxcell) and homemade (Tris egg yolk) extenders. Further, evaluate advanced methods of early pregnancy diagnosis (18^{th} to 22^{nd} days post AI) using Progesterone analysis and Ultrasonography. Post-thaw semen viability, forward motility, live percent and morphological abnormality were 67±2.6%; 43.59±3.7%; 47.22±1.8% and 42.12±1.26% for TEY preserved semen, respectively; while the same parameters were 68.11±2.6%; 40.83±4.0%; 46.52±1.7% and 41.64±1.1% for Bioxcell preserved semen, respectively. HOS test was higher in TEY (8.87±0.72) extended semen than that of BioXcell extended semen (4.45±1.20%). A total 120 animals were included for both study, 48 animals for conception rate of semen extenders (14 heifers and 34 cows) were equally grouped and inseminated (24 by Bioxcell – G-I and 24 by Tris egg yolk – G-II). First service conception rate was 50% for G-I and 70.8% for G-II. Though conception rate was higher in G-II, there were statistically non significant but higher values between the groups. Nevertheless, conception rate was higher for both heifers and cows within G-II (71.42% and 70.58% in G-II compared to 42.85% and 52.94% in G-I). From a total of 72 animals inseminated for early pregnancy detection, pregnancy was confirmed on Days 18^{th} and 22^{nd} by RMPT and serum P4 analysis 42(58.4%) and 37(51.4%) respectively, and on Day 28^{th} Ultrasonography 32 (44.5%) post AI. Although pregnancy detection with RMPT was statistically non significant but the values was higher between the tests. RMPT was the most sensitive (88.9%) but less specific (72.2%) taking Ultrasonography as a gold standard. Pregnancy later performed by transrectal palpation on Day 40 post AI showed 27 (37%) indicating the presence of corpus luteum and early embryonic mortality. RMPT was found to be relatively cheaper, and easier to apply under field condition. Results comparable to ultrasonography can be found if the test is repeated 7-8 days later. In conclusion, semen extenders can be locally prepared to mitigate the cost commercial extenders with even better conception results. Diagnosis of pregnancy can also be made at an earlier stage using a RMPT under the field conditions of Ethiopia.

Key words: *Conception rate, early pregnancy detection, Rapid milk test, serum progesterone, Ultrasonography*

1. INTRODUCTION

Semen extender is liquid diluents which are added to semen to preserve its fertilizing ability. It acts as a buffer to protect the sperm cells from their own toxic byproducts, and protects sperm cells from cold shock and osmotic shock during the chilling and shipping process (the sperm is chilled to reduce metabolism and allow it to live longer). The addition of extender to semen protects the sperm against possible damage by toxic seminal plasma as well as providing nutrients and cooling buffers if the semen is to be cooled also to protecting sperm from bacteria by adding antibiotics to it to prevent increase of bacteria (Anzar *et al.,* 2003, Arthur *et al.,* 1997).

As an alternative to replace the component of animal origin semen extenders now-a-days, commercial extenders such as AndroMed®, Biociphos plus® and Bioxcell® are used by many semen laboratories. Most of these commercial semen extenders have got soya lecithin which contains phosphatidyl choline and saturated fatty acids which maintains the structural stability of cells during cryopreservation (Oke *et al.,* 2010). However, they are not cheaply available. In Ethiopia, Andromed and Laciphos were most commonly used and are expensive as a bull semen extender by NAIC, and use BioXcell imported from IMV technologies (France). Apart from their cost, semen extended from both commercial extenders has consistently resulted in low conception rate (Anzar *et al.,* 2003).

Early diagnosis of pregnancy would help to evaluate the animal at an early date and apply alternative manipulations in the current systems of animal breeding. Furthermore, early identification of pregnant or non-pregnant dairy cows and heifers post breeding can improve reproductive efficiency and pregnancy rate by decreasing the calving interval and increase AI service rate (Omid *et al.,* 2013). Thus, new technologies to identify pregnancy or non pregnancy of dairy cows and heifers early post insemination (AI) may play a key role in management strategies to improve reproductive efficiency and profitability of dairy farms (Paul, 2010). In dairy cattle management the implementation of AI are creating the need for more accurate and timely diagnoses of pregnancy (Jensen *et al.,* 2009).

Progesterone is a hormone produced and released into the blood by the corpus luteum on the ovary. The corpus luteum is formed after the follicle has ovulated and formed on the ovary. This hormone is low during heat, and begins to rise after ovulation as the corpus luteum develops. If the cow was bred and becomes pregnant, progesterone in blood and milk remains high until just prior to calving. If the cow doesn't conceive, the corpus luteum begins to degenerate (regress) on approximately day 17th of the cycle, and progesterone declines to minimal concentrations on days 20 through 23 as the cow returns to the next cycle (NADIS, 2016). The concentration of progesterone in the milk and blood (serum) is predictive for either the animal is pregnant or not, or if any reproductive problem (Fricke, 2002).

The methods of pregnancy diagnosis are divided into direct and indirect methods or it can be divided in to visual, clinical and laboratory methods. Direct methods of PD include Transrectal palpation and Ultrasonography, this methods are accurate methods for detecting pregnancy and require a great deal of skill and experience (Paul, 2010). Indirect methods of early PD use qualitative or quantitative measures of reproductive hormones at specific stages post insemination or detect conceptus specific substances in maternal body fluids as indirect indicators of the presence of viable pregnancy (NADIS, 2016). Indirect methods include progesterone, estrone sulphate, PAG, early pregnancy factor and interferon-tau (Paul, 2010).

The potential economic benefits laboratory preparation of extenders has the advantages of lesser cost and use of fresh extender for higher conception rate, and early pregnancy detection include timely culling, saving costs, maintaining cows which will not provide economic returns and providing information to allow planning for replacement needs and shortening the calving interval through enabling the farmer to identify open animals earlier so as to treat and/or rebreed them at the earliest opportunity (Whittier *et al.,* 2013).

Therefore, the objectives of the present study were:-

- To perform post-thaw performance of semen prepared with Tris egg yolk and Bioxcell
- To evaluate conception rate of semen extended in Tris egg yolk and Bioxcell
- To compare efficiency of early pregnancy detection methods using Ultrasonography and progesterone hormone analysis in dairy cattle

2. LITERATURE REVIEW

2.1. Extenders for semen preservation

Semen extender is liquid diluents which are added to semen to preserve its fertilizing ability. It acts as a buffer to protect the sperm cells from their own toxic byproducts, and protects sperm cells from cold shock and osmotic shock during the chilling and shipping process (the sperm is chilled to reduce metabolism and allow it to live longer). The extender allows the semen to be shipped to the female, rather than requiring the male and female to be near to each other (Amirat *et al.,* 2004*).* Special freezing extender use also allows cryogenic preservation sperm ("Frozen semen"), which may be transported for use, or used on-site, at a later date (Anzar *et al.,* 2003*).* Semen extenders should not be confused with drugs or nutritional supplements designed to increase the volume of semen released during an ejaculation (Arthur *et al.,* 1997).

The addition of extender to semen protects the sperm against possible damage by toxic seminal plasma as well as providing nutrients and cooling buffers if the semen is to be cooled also to protecting sperm from bacteria by adding antibiotics to it to prevent increase of bacteria. In the case of freezing extenders, one or more penetrating cryoprotectants will be added (Mahmoud *et al.,* 2013). Typical cryoprotectants include, glycerol, DMSO, dimethyleformamide. of semen extenders for preservation and storage of quality semen for higher conception rate (Siddique *et al.,* 2006). This has been found to result in insemination of good quality semen for normal conception, fetal development and offspring (Siddique *et al.,* 2006). The process of artificial insemination needs an experienced expertise and quality frozen semen for successful conception of the animals (Morrell*,* 2011).

The method of preservation ideally enables the semen to be stored for an unlimited period and availability of genetic material from superior bulls without geographical limitation *(*Morrell, 2011*).* The failure to properly evaluate the semen would render the risk of passing over poor genetic material to a large population. The success of AI operation largely depends on the quality and fertility of semen that has been rigorously evaluated and passes through routine and correct process of cryopreservation (Gebremedhin, 2008). Furthermore, artificial insemination is one of the most important tools for genetic improvement in modern dairy cattle breeding practices and it is only possible with efficient extender. Extender has a vital role in the preservation of sperm cell and its quality parameters such as viability, motility, acrosomal and membrane integrity etc. Semen extenders contain buffering system to maintain pH of the medium (Tris, sodium phosphate, citric acid), cryo shock preservatives (glycerin, egg yolk, soy-lecithin, milk), provide energy (fructose) and guarantee the microbial free environment i.e. antibiotics (streptomycin, penicillin, polymixin B) (Rehman*, et al.,* 2014).

Quality semen extender provide energy for metabolic activities within sperm cell; maintain osmotic pressure and pH of the medium (Salamon and Maxwell, 2000). Extender also keeps a check on the contamination of the medium to protect semen from microbial growth. Different semen extenders provide sufficient nutrition in the form of fructose sugar to sperm cells during storage. It also prevents sperm cells against cryo shocks during cold storage at extreme temperature in liquid nitrogen (-196ºC). Moreover, liquid extended frozen semen produces a higher conception rate with a relatively less number of sperm cells (Anzar *et al.,* 2003).

2.1.1. Bioxcell Semen Extender

Bioxcell is a commercial ready produced and imported from IMV technologies (France) to use preparation only diluted with bi-distilled water. BioXcell innovative animal protein-free formula eliminates the health risks associated with the use of milk and/or egg yolk for the final dilution of bovine semen. The preparation is in a 250ml bottle which is stored at +2 to +8 degrees. Media for frozen (-196°C) or fresh semen (+4°C). Semen diluted in Bioxcell can be processed at refrigeration temperature (+4ºC) or room temperature (+20ºC). It has been proven to maintain good fertility in semen doses containing low concentrations of sperm cells (Andrabi *et al.,* 2008).

Bioxcell contains an antibiotic mix of Lincomycin, Spectinomycin, Gentamycin and Tylosin. Ochre colored, this sterile concentrated liquid is presented in 100 ml PET biocompatible, unbreakable bottle. Easy to use, 1 bottle of Bioxcell is diluted in 400 ml of water for a final extended, ready to use volume of 500 ml. Due to the absence of egg yolk and/or milk, Bioxcell is clear after dilution. This makes diluted semen easy to evaluate under the microscope or CASA System (Rodríguez, 2003). Bioxcell may be frozen at -20ºC after extension. The bottle will be kept in a water bath at 34 degrees for 10 minutes before use. About 1000ml of bi-distilled sterile, pyrogen-free water will be placed in Erlenmeyer or big flask and kept in a water bath at 34 degrees for 10 minutes. Then the extender will be slowly added to the pure water, the content will be rinsed once or twice and thoroughly mixed (Oke *et al.,* 2010*).*

2.1.2. Tris Egg Yolk Semen Extender

Egg yolk has been used as a basic component of extenders for bull ejaculate since 1939 (Amirat *et al.,* 2004). Though addition of egg yolk, changes the composition of an extender, it still is recommended because of the excellent protection effect that it provides on sperm cells. It contains a low density lipoprotein which has a cryoprotective effect on the integrity of plasma membrane as well as on the percentage of normal spermatozoa and sperm motility. In spite of all the benefits of egg yolk on semen cryopreservation, sometimes it serves as a potential source of microbiological contamination which compromises the quality of cryopreserved semen and its standardization. Hence, world organization for animal health (OIE) in 2003, recommended that the products of animal origin used in semen processing should be free of any biological risk (Celeghini *et al.,* 2008).

Therefore as an alternative to replace the component of animal origin semen extenders now-a-days, commercial extenders such as AndroMed®, Biociphos plus® and Bioxcell® are used by many semen laboratories. Most of these commercial semen extenders have got soya lecithin which contains phosphatidyl choline and saturated fatty acids which maintains the structural stability of cells during cryopreservation (Oke *et al.,* 2010*).* However, they are not cheaply available. In Ethiopia, Andromed and Laciphos were most commonly used as a bull semen extender by the National Artificial Insemination Center (NAIC) and very recently NAIC has changed both for BioXcell imported from IMV technologies (France).

Apart from their cost, semen extended from both commercial extenders has consistently resulted in low conception rate. It has not been confirmed whether the freezing protocol played a role in this, however the role of these semen extenders has been previously described. Preparation of extenders at laboratory has the advantages of lesser cost and use of fresh extender. Tris-egg yolk extender was prepared by mixing 2.42 g Tris hydroxymethyl amino methane, 1.48 g citric acid, and 1.0 g fructose in 100 ml of bi-distilled water. The mixture is boiled for 20 minutes until boiling. The boiled mixture was allowed to cool and 25 mg Gentamycin, and 50,000 IU penicillin was added and thoroughly mixed. Finally 6.4 ml glycerol, 20 % v/v of chicken egg yolk was added. Once egg yolk is added, the extender must be stored at -20 for longer period of storage and before usage it must be thawed to 37degrees (Berran *et al.,* 2012).

2.2. Effect of extenders on semen quality and pregnancy success

Semen quality is critically important for the success of AI and semen can be most useful for AI if it can be cryopreserved and extended with quality semen extenders for successful reproduction. Since this method of preservation ideally enables the semen to be stored for an unlimited period and availability of genetic material from superior bulls without geographical limitation (Morrell, 2011). Hence, failure to properly evaluate the semen would render the risk of passing over poor genetic material to a large population. The success of AI operation therefore largely depends on the quality and fertility of semen that has been rigorously evaluated and passes through routine and correct process of cryopreservation (Gebremedhin, 2008).

Although there are some auspicious achievements of AI the success rate was not as expected because it has been constrained by a number of factors including technical, system related, managemental and financial problems (Belihu, 2002; Shiferaw, *et al.,* 2003; Gebremedhin, 2008; Demeke, 2010). Further, every step of an ejaculate processing starting from collection to production of AI doses is very important and can affects final semen quality. Out of all steps involved in semen processing, dilution of sperm, filling of straw, their cooling and freezing for producing frozen semen doses, have significant effect on sperm motility and the effect of the extender used is especially important (Siddique *et al*., 2006). Semen extenders protection for sperm membrane and structures function with higher level of cryo preservation and fertility. Semen extenders led to increased motility and integrity of plasma membrane. Better development of semen extenders for enhancing post-thawed quality bull spermatozoa has an impact on conception rate.

Semen extender should provide energy for metabolic activities within sperm cell; maintain osmotic pressure and pH of the medium (Salamon and Maxwell, 2000). Extender also keeps a check on the contamination of the medium to protect semen from microbial growth. Different semen extenders provide sufficient nutrition in the form of fructose sugar to sperm cells during storage. It also prevents sperm cells against cryoshocks during cold storage at extreme temperature (-196^{o}C) in liquid nitrogen. Moreover, liquid extended semen produces a higher conception rate with a relatively less number of sperm cells (Anzar *et al.,* 2003).

2.3. Methods of Pregnancy Diagnosis in Dairy Cattle

Benefits and drawbacks of early pregnancy detection

Early pregnancy diagnosis has become a key to economic success in cattle production and confirming pregnancy is essential for improving fertility management strategy. Early identification of pregnancy and non pregnant in dairy animals post breeding can improve reproductive efficiency and pregnancy rate. Accurate early detection of pregnant and non-pregnant cows is an essential factor for optimizing reproductive performance in dairy cattle. In dairy cattle, pregnancy diagnosis is an important tool to measure the success of a reproductive management. The assessment of pregnancy diagnosis can be conducted at varying intervals after service. The methods used can have varying degrees of accuracy.

However, pregnancy diagnosis only gives an assessment at a particular point of time, after which undetected loss of pregnancy may occur and give a false result. A wide variety of methods have been used and each has its own advantage and disadvantages. Early diagnosis of pregnancy is essential for profitable animal husbandry, especially in the productive animal species and crucial to shorten the calving interval through enabling the farmer to identify open animals earlier post insemination so as to treat and/or rebreed them at the early opportunity. Different methods have been evaluated and developed during the past and recent year, some of which have some limitations to their wide scale use and accuracy (Santos *et al.,* 2004).

Generally, the methods of pregnancy diagnosis are divided in to direct and indirect method or it can be divided in to visual, clinical and laboratory methods. Direct methods for early pregnancy diagnosis of dairy cattle involve direct detection of the tissues and/or associated fluids of the conceptus either manually or via electronic instrumentation. Currently available direct methods for diagnosis of pregnancy include Transrectal palpation and Transrectal Ultrasonography and both methods are currently used by bovine practitioners to diagnose pregnancy in dairy cattle.

Because they are direct methods, the test outcome can be subjective among practitioners. The technical expertise, operator proficiency and the record stage for post breeding that the technique is performed can affect the specificity and sensitivity of the test (Paul, 2010).

Indirect methods for early pregnancy diagnosis use qualitative or quantitative measures of reproductive hormones at specific stages post Insemination or detect conceptus specific secretion in maternal body fluids as indirect indicators of the presence of a viable pregnancy. Currently available indirect methods of pregnancy diagnosis include measurement of endocrine hormones such as progesterone, estrone sulphate and pregnancy specific proteins such as pregnancy-associated glycoprotein's (PAG), early pregnancy factors and interferon-tau (Paul, 2010).

The visual method of pregnancy diagnosis includes non-return to estrus, met estrous hemorrhage and exposure to a bull or artificial insemination (Paul, 2010). An appropriate method for pregnancy diagnosis depends on the management system of the reproductive program and considerations that are unique to each individual farm (MattLucy *et al.,* 2011).

2.3.1. Transrectal palpation

Palpation of the reproductive tract through the rectal wall has been the customary method for pregnancy diagnosis and mostly practiced by AI technicians (Youngquist, 2006). Rectal examination of cows is a very time-honored technique for the diagnosis of pregnancy. The proximity of the reproductive tract of the cow to the rectum and its elasticity allows a trained practitioner to detect characteristics of the reproductive tract that coincide with either pregnancy or non-pregnancy (Whittier *et al.,* 2013). Currently, rectal palpation is the easiest, fastest, cheapest and most accurate method that an experienced expertise performed (UGA, 2013).

The golden rules of rectal pregnancy examination includes examination of the entire tract before declaring the cow is open and find one of the positive signs of pregnancy before you call a cow is pregnant (Purohit, 2010). If you are not sure, recheck the cow with different methods.

The only positive signs of pregnancy in the cow are fetus, cotyledons/caruncles; amniotic vesicle and fetal membrane slip (Whittier *et al.,* 2013). Technique of pregnancy diagnosis by rectal palpation of a retractable uterus, after retracting the uterus fully and accurately, first feels the uterus for asymmetry. At 35 days of pregnancy the pregnant horn will feel slightly larger (Purohit, 2010).

Non-pregnancy is determined by a thorough examination of the uterus, usually after it is retracted onto the floor of the pelvis and the absence of the cardinal signs of pregnancy is carefully ascertained (Whittier *et al.,* 2013). Rectal palpation also, allows an estimation of the stage of pregnancy. The pregnancy status was diagnosed and days in gestation of each cow were determined by per-rectal palpation 60 days after AI (Whittier *et al.,* 2013). The stage of gestation can be estimated on the basis of palpable structures and characteristics of the uterus and fetus.

In early pregnancies, stage of gestation can be estimated on the basis of the size of the pregnant horn asymmetry and the size of the amniotic vesicle. In more advanced pregnancies, age of the fetus is estimated based on determination of the size of the fetus, fetal crown-to-nose length and position of the uterus (Youngquist, 2006). But sometimes early embryonic loss and pregnancy can be terminated by manual rupture of the amniotic vesicle due to miss handling practice in dairy cattle. Transrectal palpation induces iatrogenic embryonic mortality (Paul, 2010). The risk of embryonic mortality is high during the period of gestation when cows are diagnosed by transrectal palpation (Purohit, 2010).

Table 1: Calf fetal size at various stages of pregnancy in relation to the size of some commonly known adult animals at rectal palpation

Stage of pregnancy	**Fetal(calf) size**
2 months	Mouse
3 months	Rat
4 months	Small cat
5 months	large cat
6 months	Beagle dog

(Source: Hansen, 2008)

Detectable findings during rectal Palpation of pregnancy

The definite signs of pregnancy in the cow as determined by rectal palpation are palpation of the amniotic vesicle, slipping of the fetal membranes, palpation or ballottement of the fetus and palpation of the placentomes (Purohit, 2010).

The Amniotic Vesicle: The amnion is a portion of the placenta that contains the developing conceptus and the amniotic fluid, is palpable as early as 28 days after conception in heifers and by 32 to 35 days in older cows. The vesicle is recognized as nearly spherical, turgid, fluid-filled structure that is approximately 1 cm in diameter at 28 days and increases in size as pregnancy advances. Intentional rupture of the amniotic vesicle has been used in the past as a method to intentionally provoke abortion in cattle (Youngquist, 2006). The amniotic vesicle can be palpated with due care between 30-50 days of gestation as a movable oval object within the uterine lumen (Purohit, 2010).

Slipping of the Fetal Membranes: It is possible to feel the slipping of the chorioallantoic membrane (fetal membrane) along the greater curvature within the uterus (NADIS, 2016). The fetal membrane slip can be felt between 35-90 days of gestation (Purohit, 2010). The examiner can detect the chorioallantois (developing placenta) within the lumen of the pregnant uterus by compressing the uterine horn between the thumb and forefinger, lifting the uterus and then allowing the horn to slowly “slip” from the grasp. If the cow is pregnant, the chorioallantois can be felt to slip through the fingers just prior the uterine wall. The membranes can be slipped in the pregnant horn as early as 30 days and can be reliably detected by day 35. During early pregnancy, the fetal membranes are thin and a delicate touch and some experience are required to recognize this sign of pregnancy (Youngquist, 2006).

Palpation of Placentomes: Placentomes are the structures formed by the union of maternal caruncles and fetal cotyledons by which the placenta is attached to the uterus (NADIS, 2016). The presence of placentomes is another positive sign of pregnancy and is detectable from about 75 days to term. The period of pregnancy when the uterus has descended into the abdominal cavity and the fetus is not palpable, palpation of a placentome is the surest indication that the cow is pregnant. In general, they can be detected as soft, thickened lumps in the uterine wall and are more easily detected as pregnancy advances (Purohit, 2010). The size of placentomes varies with the stage of gestation and their location in the uterus. They are most consistent in size just in front of the cervix and are palpated at that location to estimate the stage of gestation (Youngquist, 2006).

Palpation of the Fetus: As pregnancy progresses, it becomes possible to feel the presence of the fetus within the pregnant horn. After about day 150, the fetus is too far forward in the body cavity to palpate the entire fetus although fetal structures can be palpated (NADIS, 2016).
The palpation of the fetus itself is a positive sign of pregnancy. Depending on the skill of the examiner and the location of the fetus, the fetus can be palpated from the time of amniotic softening (65 to 70 days) to term. The whole of the fetus is palpable many times only during early gestation (2 to 4 months) (Purohit, 2010). In the early stages, the fetus can be grasped directly. Later, the fetus is detected by ballottement; the examiner sets the fetal fluids in motion by rocking the hand against the uterine wall and recognizes the fetus as it rebounds against the hand (Purohit, 2010).

The fetus is identified as a free-floating firm object within the fluid-filled uterus during the first 4 months of gestation. As pregnancy advances, increased weight of the fetus and fluid pulls the uterus downward and forward until the fetus comes to rest on the abdominal floor during the fifth and sixth months. Continued growth of the fetus positions it closer to the maternal pelvis during the last trimester and palpation of the fetus is facilitated (Youngquist, 2006).

Estimation of the stage of pregnancy is performed by rectal palpation and days in gestation of each cow were determined (Whittier *et al.*, 2013). The size of the fetus as explained in the table below is approximately that of a mouse or rat at 2 and 3 months and it increases to the size of a small cat at 4 months, a large cat at 5 months and a beagle dog at 6 months respectively. Beyond 8 months of gestation, fetal parts (legs, head) are palpable within the pelvic cavity or just cranial to the pelvic brim. Palpation of a fetal extremity is sufficient evidence for pregnancy if other uterine findings are normal (Purohit, 2010).

Table 2: Developmental changes of conceptus during early embryonic period

Days post fertilization	Developmental changes
Days 0-1	Fertilization, single-cell embryo (zygote) in oviduct
Day 2	Early cleavages in the oviduct (up to 8 cell stages), activation of Embryonic genome
Days 3-4	Embryo enters the uterus
Days 5-6	6–32 cell zona-enclosed embryo progressing into compact morula Stage
Days 7-8	Formation of a blastocoels with differentiation of embryonic cells
Days 9-10	Blastocyst elongation from a tubular to a filamentous structure
Days 11-13	Maternal recognition of pregnancy
Days 14-19	Implantation begins
Days 19-20	Caruncles-cotyledons appear
Days 21	Implantation progresses
Days 22-41	Implantation completed
Days 42	Implantation completed

Source: Morris and Diskin, 2008

2.3.2. Advanced Methods of Pregnancy Diagnosis in Dairy Cattle

Ultrasonography

Pregnancy diagnosis using Transrectal Ultrasonography, an experienced expert can accurately diagnose the animal only after day 35 of gestation, but the application of Ultrasonography has made diagnosis possible as early as day 25 - 28 after insemination or even earlier (Sharma, *et al.*, 2011). Transrectal Ultrasonography provides additional information on ovarian structures, identification of twins and determination of fetal viability, age and sex (Fricke, 2002), (Sharma, *et al.*, 2011), (Ali and Fahmy, 2008). Transrectal Ultrasonography made a thorough examination of the reproductive health of the animal possible and, therefore, it has now become an established research tool to study bovine reproductive physiology in cattle (Fricke, 2002). It is a direct, accurate and real time method for pregnancy diagnosis. The outcome of the test is known immediately at the time the test is conducted. It requires a great deal of skill and experience to read the ultrasound. Transrectal Ultrasonography is slowly being incorporated into reproductive management schemes in dairies primarily by bovine practitioners who have adopted this technology (Fricke, 2002).

The extent to which Transrectal Ultrasonography will displace Transrectal palpation as the primary direct method for pregnancy diagnosis in dairy cattle remains to be seen; however, if current trends continue, Transrectal Ultrasonography may displace Transrectal palpation as the direct method of choice for pregnancy diagnosis among bovine practitioners. Transrectal Ultrasonography reduces the interval from insemination to pregnancy diagnosis by 10 to 15 days compared to rectal palpation (Fricke, *et al.*, 2003). Accuracy over 99% can be achieved, enabling fertility problems to be identified rapidly. Two factors affect the speed at which ultrasound examination can be conducted in dairy farm: operator proficiency, availability and restraint of animals. When both factors are optimized, the speed of Ultrasonography approach that of rectal palpation, while exceeding rectal palpation in the amount of information gathered from each animal. Pregnancy can be detected earlier with ultrasound compared with rectal palpation (NADIS, 2016).

In veterinary ultrasound machines equipped with a rectal transducer are expensive in developing countries and therefore the high initial cost of this technology partly limits its practical implementation (Fricke, 2002). The main disadvantages of the use of Ultrasonography are related to cost and time involved with the use of this technique. Ultrasound machines are expensive and it takes more time to perform a pregnancy diagnosis with an ultrasound machine than by rectal palpation (NADIS, 2016).

Serum Progesterone analysis, Rapid milk Progesterone Pregnancy test

Progesterone has been called "the hormone of pregnancy" due to its basic part in supporting the endometrium (uterine covering) and thus the survival of the conceptus. Progesterone is a hormone produced and released into the blood by the corpus luteum on the ovary. The corpus luteum is formed after the follicle has ovulated. This hormone is low during heat, and begins to rise after ovulation as the corpus luteum develops. If the cow was bred and becomes pregnant, progesterone in blood and milk remains high until just prior to calving. If the cow does not conceive, the corpus luteum begins to degenerate on approximately day 17 of the cycle, and progesterone declines to minimal concentrations on days 20 through 24 as the cow returns to heat. The concentration of progesterone in the blood (serum) is correlated closely with the concentration in milk. In fact, since progesterone is a steroid hormone, it has an affinity for milk fat; thus progesterone in milk is somewhat higher than in blood. However, the relative relationship between milk and blood levels is the same. Variation in the concentration of progesterone in milk due to stage of the cycle or pregnancy status is much greater that the effect caused by the variation in fat content (Fricke, 2002).

Rapid progesterone detection kit that was used for the early identification of pregnant and non pregnant animals on day 18-24 post insemination as per the producer's instruction**.** It is a qualitative test indicated by the test kit that is based on the presence of Progesterone hormone in the milk. The test kit indicate the presence of progesterone in the milk displayed on the stick by visible control and test lines and predict the absence of pregnancy and reproductive abnormalities if any, this is developed by employing highly sensitive technique of Enzyme linked Immuno Sorbent Assay (ELISA). It is a commercially available user friendly test kit and does not require any kind of laboratory infrastructure.

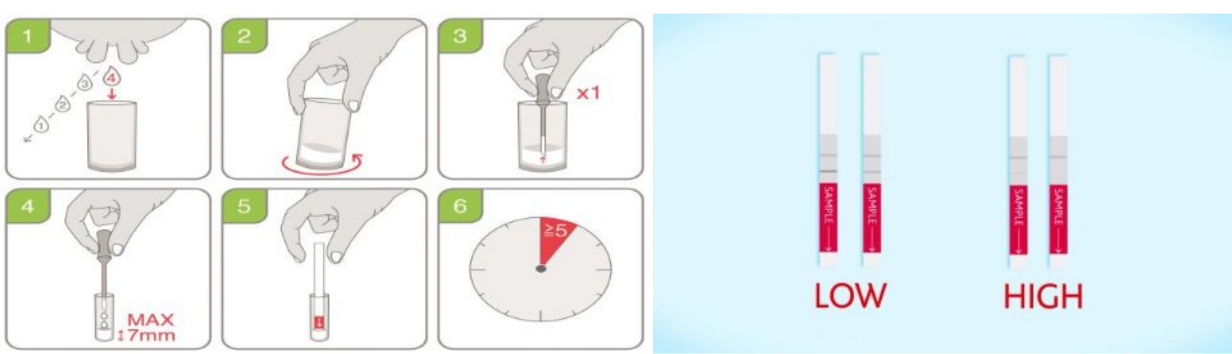

Figure 1: Test procedure of rapid milk progesterone test kit

Key: *1= Prepare a collection pot & collect only from one quarter 2= Mix well the milk to distribute the fat, 3= draw up some milk to the base of the bulb, 4= Transfer the milk to a test tube to a maximum depth of 7mm., 5= open the P4 kit and remove the stick, label and with the arrows pointing down, put the stick in the test tube, 6. Wait 5 minutes for the result to show on test and control lines.*

Measurement of progesterone is an indirect method for pregnancy diagnosis in many livestock species including cattle. Conception extends the life of the corpus luteum (CL) by preventing the luteolytic mechanism from being triggered, thus prolonging and maintaining its functional characteristics, insuring continued high progesterone levels. Progesterone maintains the uterine endometrium in a state which supports embryonic development, implantation and foetoplacental development (Balhara, *et al.,* 2013). The corpus luteum formed on the ovary subsequent to ovulation produces progesterone for maintenance of pregnancy for entire gestation in cow. In normally cycling cows the CL is lysed because of the effects of prostaglandins from the uterus if the animal is not pregnant and thus the progesterone level goes down. Therefore, low progesterone concentrations in maternal milk or blood at 18 to 24 days post breeding can predict that the animal is non-pregnant and high progesterone gives an insight that probably the animal is pregnant (Purohit, 2010).

Progesterone concentrations in milk or serum can be quantified using a laboratory RIA or ELISA procedures (Paul, 2010). Currently, milk progesterone is the only test that is likely to be a competitor to Ultrasonography and rectal palpation. A single sample taken 18 - 24 days post insemination will accurately identify non-pregnant cows; > 95% of cows with low progesterone will not be pregnant. However, a significant proportion of cows that are non-pregnant will have high progesterone 24 days after AI, so with a single milk progesterone test around 20 to 25% of cows identified as pregnant will not be (NADIS, 2016). Although subtle but, significant differences in serum progesterone concentrations have been reported between pregnant and non-pregnant cows early post breeding (Mann, and Lamming, 2001), (Gümen, *et al.,* 2003), assessment of plasma progesterone at 18 - 24 days post Artificial Insemination was an accurate method for identifying non pregnant cows (Gümen*, et al.,* 2003).

The main advantage of using milk or plasma progesterone concentrations to diagnose pregnancy is that, this method allows detection of pregnant and non-pregnant cows soon post insemination. In particular, cows can be diagnosed for pregnancy as early as 18 to 24 days post insemination. There are several disadvantages of using milk or plasma progesterone to diagnose pregnancy. Milk and plasma progesterone samples need to be collected at least two times after insemination. Usually, samples are collected between 18 to 24 days post insemination. If either one of those samples is considered to have low progesterone concentrations, cows are diagnosed as not pregnant. If both samples have high progesterone concentrations, however, there is still a certain probability that the cow is not actually pregnant. The reliability of progesterone tests to detect pregnant cows is estimated to be only around 80%. That means that 20 % of cows diagnosed pregnant by progesterone are, in reality, not pregnant but suspected to be (Muhammad, 2009).

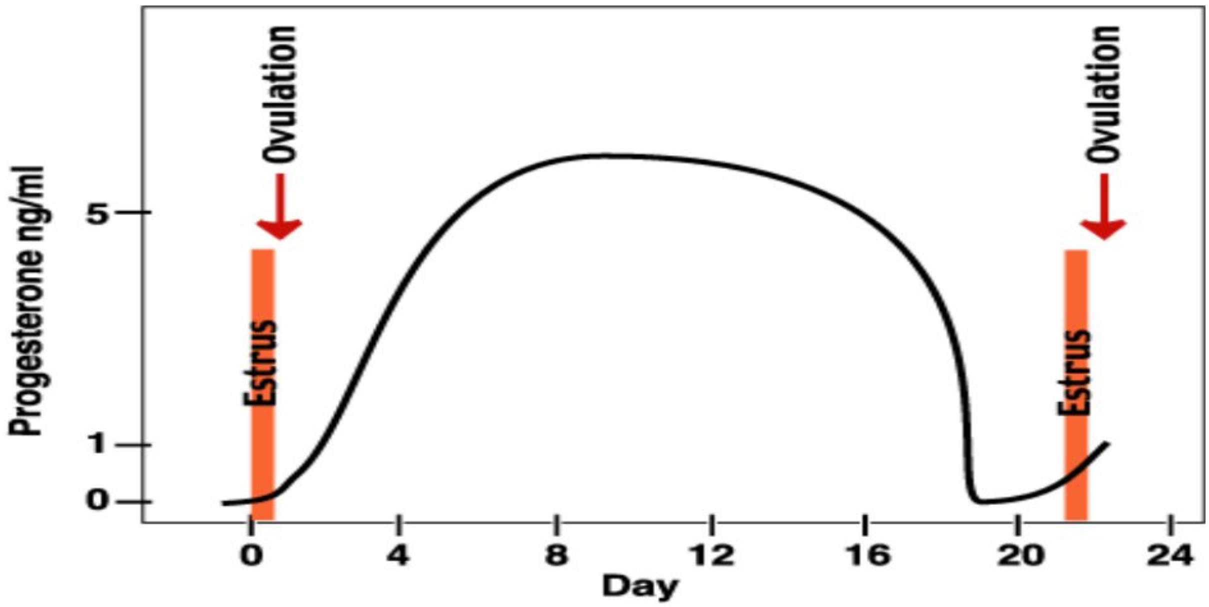

Figure 2: Progesterone concentration profile during a normal estrus cycle in bovine

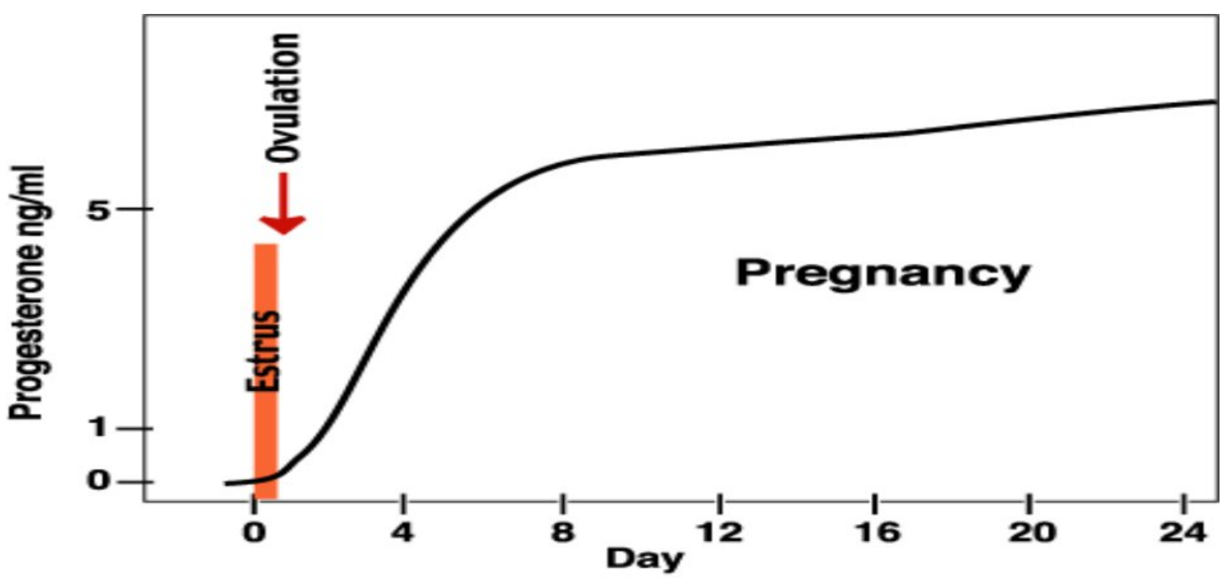

Figure 3: Serum progesterone profile during pregnancy in bovine

Determination of Metabolites and Other Steroid Hormones

Estrone sulphate

Estrone sulfate is a conjugated estrogen that has been used to diagnose pregnancy using milk samples in cattle. Estrone sulfate is produced specifically by the conceptus (embryo-fetus and associated placental membranes) and, therefore, its presence is a direct indicator of pregnancy (Paul, 2010). It is a conjugated steroid product of Estrone, present predominantly in the bovine placentomes and it is the major Estrone present in the fetal (allantoic and amniotic) fluids and maternal peripheral plasma of cows (Balhara, *et al.,* 2013). In cows, concentrations of estrone sulfate detectable in the whey fraction of milk are similar to those in maternal plasma and increase from about 60 days in gestation to the end of lactation. However, because concentrations of Estrone sulfate in maternal circulation are not reliably detectable until around 80 days in gestation, Estrone sulfate cannot compete with progesterone to assess pregnancy status early post breeding in cattle and therefore this test can only detect late pregnancy (Paul, 2010; Balhara, *et al.,* 2013).

Estrone sulphate is produced by the foetal-maternal unit and present in milk and plasma of non-pregnant animals, as well, but its concentration increases as pregnancy advances. Several studies report that after day 100 to 120 of gestation a high proportion of pregnant cows will have Estrone sulphate levels above the highest concentration found in non-pregnant animals (Hirako, *et al.,* 2002). Concentration of Estrone sulphate in the maternal body fluids is a useful indicator for the placental functions especially those related to embryonic growth. Estrone sulphate concentrations have also been frequently correlated to fetal numbers (Lobago, *et al.,* 2009). These are higher when the number of developing fetuses is more than one and yet, Estrone sulphate is not an ideal pregnancy biomarker as the plasma and milk profiles are influenced by many other factors such as genetic makeup, weight, parity status and environment (Telugu, *et al.,* 2009).

Pregnancy associated glycoprotein (PAG)

The pregnancy associated glycoprotein's (PAGs) are secretary products from the mono and binucleated trophoblastic cells in bovine placentomes (Balhara, *et al.*, 2013). The PAGs consist of a large family of more than 20 closely related proteins that are only produced by the placenta (Green, *et al.*, 2005). They can be detected in the blood of pregnant cows beginning at approximately 25 days after insemination (Matt Lucy and Scott Poock, 2012). Monitoring the concentrations of PAGs in blood is an effective method of pregnancy detection (Sousa, *et al.*, 2002). By using biochemical procedures, some molecules of the PAGs were isolated from cotyledons of cow (Klisch, *et al.*, 2005; Xies, *et al.*, 1991).

Although their function during pregnancy or parturition is unclear (Zoli, *et al.*, 1992) demonstrated that PAGs are inactive members of the aspartic proteinase family. If enzymatically active, PAGs could be important in structural remodeling of the placenta during pregnancy or in placental detachment after parturition (Rahul, *et al.*, 2012). Although PAG are presently one of the more promising markers for early detection of pregnancy in cattle, PAG-based pregnancy tests are limited by a number of factors. First, PAG is not detectable in milk or urine and therefore requires that a blood sample be collected, a procedure that is difficult on some farms. Second, because PAG reaches high concentrations in maternal circulation during the periparturient period and has a long serum half-life, cows inseminated too early postpartum are not eligible for testing (Paul, 2010).

Early conception factors

Early pregnancy factor (EPF, also known as early conception factor-ECF) which is present in the sera of pregnant mammalian females, detectable within 6 to 24 hours of fertilization and disappearing within 24 to 48 hours after death or removal of the embryo. EPF remains the earliest serum benchmark for positive fertilization and hence successful conception. Early pregnancy factor (EPF) is a protein that was first detected in the serum of pregnant mice within 4 to 6 hours after mating (Balhara, *et al.*, 2013). EPF is made of two components (EPF-A and EPF-B). EPF-A is secreted by the oviduct as well during gestation but EPF-B is secreted by the ovary. Production of EPF-B requires a signal from the fertilized egg (ovum factor).

EPF is an attractive marker for pregnancy in that it appears within hours after conception and disappears rapidly after death or removal of the embryo (Youngquist, 2006; Roberts, 2007). Serum concentrations of the various EPF forms and their components vary from species to species and are dependent on gestational age (Roberts, 2007). It is recommended that milk or serum samples be tested at 7 days after insemination. This molecule was supposedly present in the blood of pregnant cattle within two days after conception. The exact nature of this molecule and how it got into circulation were not well defined but nonetheless it could be assayed by using a rosette inhibition test (Purohit, 2010).

Interferon tau

Bovine interferon tau (BoIFN- tau) is one of the principal proteins secreted by the bovine conceptus from 16 to 25 days. Interferon-tau, a novel type I interferon, is produced in large amounts by the embryo after day 14 to signal the mother and establish the pregnancy (Bazer, 2013). It is the pregnancy recognition hormone in cattle and other ruminants that acts to silence expression of estrogen receptor alpha (ESR1) and, in turn, oxytocin receptor (OXTR) to prevent development of the luteolytic mechanism that required oxytocin (OXT) from the corpus luteum (CL) and posterior pituitary to induce luteolytic pulses of PG F2α. Thus, IFN-tau blocks the ability of the uterus to develop the luteolytic mechanism (Wolf, *et al.,* 2003; Bazer, *et al.,* 2011).
A primary function of IFN-tau is to abrogate development of the uterine luteolytic mechanism to ensure maintenance of ovarian CL to produce P4, the hormone essential for establishment and maintenance of pregnancy (Oliveira, *et al.,* 2008). IFN-tau, is acting within the uterine cavity (Balhara, *et al.,* 2013). With extremely low levels in extra uterine tissues and peripheral circulation, prevents direct use of IFN-tau as an early pregnancy diagnosis molecule (Janaway, *et al.,* 2008). The INF-tau secretion is transitory. It reaches a maximum by 20 to 24 days and is completely gone by day 30 of pregnancy. The IFN-tau is unlike hCG because its expression is transitory and it does not accumulate in the blood or urine. Thus, IFN-tau cannot be used for a pregnancy test in the blood or urine of the cow (Sousa, *et al.,* 2002).

Table 3: Summary of selected references for reports of pregnancy diagnosis using rapid milk progesterone test in Holstein Frisian cows

Reference	Days of test post AI	Pregnant [%]	Non Pregnant [%]
Glatzel *et.al.,* 2000	19-23	87.8	91.4
Muhammed *et. al.,* 2000	21-24	83.3	100
Otava *et. al.,* 2007	19	82	94
Nakhashi *et. al.,* 2010	18-24	86.2	92.1
Shelar *et. al.,* 2011	22	87.5	100
Barbato and Barile, 2012	21	75	83
Gaja *et. al.,* 2013	18, 21, 23	85	92
Naikoo *et. al.,* 2013	20-23	84	89
Romagnolo & Nobel, 1993	21	84.7	92.3
Kaul and prakash, 1994	20, 22, 24	86.6	100
Lec *et. al.,* 1996	17, 19, 21	82/87	86/96
Gupta *et. al.,* 1991	18-24	71.43	81/82
Lackchawura *et.al.,* 2003	21	90/92	100
Samad *et. al.,* 2004	19-21	87.8	960
Ucar *et. al.,* 2004	19-24	81	1000

***Key**: P- Pregnant, NP- Non pregnant, AI- Artificial insemination*

3. MATERIAL AND METHOD

3.1. Study Area

The study was conducted from November 2018 to May 2019 in private and government owned dairy farms in Bishoftu (Adea) and Modjo (Lume). Bishoftu is located at 9°N and 40°E, in Oromia National Regional State about 47 km southeast of the capital city of Ethiopia, Addis Ababa. The altitude is about 1850 m. a. s. l. It experiences a bimodal pattern of rainfall with the main rainy season extending from June to September (of which 84% of rain is expected) and a short rainy season from March to May with an average annual rainfall of 800 mm. The mean annual minimum and maximum temperatures are 12.3 and 27.7ºC, respectively, with an overall average of 18.7°C. The highest temperatures recorded in May and the mean relative humidity is 61.3% (CSA, 2010).

Modjo is the administrative center of Lume district, located in the East Shewa Zone of the Oromia Region, Ethiopia. It is located at 66 Km South-east of Addis Ababa and lies at latitude 8°35'N and longitude 39°7'E at an altitude of 1790 meters above sea level. The area gain rainfall twice a year those known as long and short rainy season. The main rainy season extends from June to September. The average annual rainfall, temperature, and mean relative humidity are: 776mm, 19.4 ºC and 59.9% respectively (CSA, 2010).

3.2. Study population

Study animals were randomly selected 120 cross breed dairy cattle (heifers and cows) owned by private farmers. The study was conducted in two phases, 48 (14 heifers and 34 cows) animals were used for evaluation of conception rate while 72 animals were used for pregnancy diagnosis and heifers were excluded from this study because of milk sample test. All animals were subject to clinical and gynecological examination of the reproductive tracts to check for abnormalities and to determine the reproductive status of individual animals using trans-rectal Ultrasonography. Normally cycling and apparently healthy animals without any reproductive disorder from their history of record book were selected randomly as a candidate.

3.3. Study Design

3.3.1. Evaluation of record of semen analysis & laboratory examination of post-thaw sperm parameters

A record of 39 semen collections from 10 breeding bulls maintained at NAIC, Kaliti was considered. Records of semen parameters mass motility [Score], Progressive forward motility [%], Live percent [%] and Morphological abnormality [%] were taken. Straws that were packed at a concentration of 120mill/ml as per the standard of the National Artificial Insemination Center were collected from CVMA reproduction laboratory for post-thaw evaluation. Cryopreservation procedures were as per the conventional method applied at the center. Semen samples (about 2 ml from each bull) was extended each in Bioxcell and Tris egg yolk extenders and filled in a 0.25ml straw, sealed, equilibrated for 4 hours at +4 degrees. Straws were then stored in a liquid nitrogen at -196 degrees. The straws from each extender were withdrawn, thawed at 37 degrees for 30 seconds and evaluated for post-thaw motility, live percent, morphological abnormality and hypo-osmotic swelling test.

Progressive motility, morphological abnormality and post-thaw live-dead percent were performed in a similar fashion as for the fresh semen sample. For Hypo-osmotic Swelling Test (HOST), 1 ml pre-incubated (at 37ºC) HOST solution (prepared from 7.35 g sodium citrate tri hydrate, 13.51 g fructose in 1,000 ml of distilled water having 150mOsm/L) was mixed with 0.1 ml of extended semen in a test tube. Simultaneously, equal volume of control solution prepared from phosphate buffer saline was mixed with 0.1 ml of same semen in another test tube. The test tubes were incubated for 60 minutes at 37°C. After 1hr 0.1 ml of 10% formalin was added for fixation of spermatozoa. A drop of incubated semen was examined at 1000X magnification and observed for curling pattern of sperm tails. The proportion of swollen spermatozoa was determined by method given by (Sharma *et al*., 2012)

Host reactive spermatozoa (%) = % swollen spermatozoa in HOS solution - % swollen spermatozoa in PBS.

3.3.2. Evaluation of conception rate with frozen semen preserved in Bioxcell and Tris egg yolk extenders

A design for both studies was experimental. A total of 48 animals (14 heifers and 34 cows) were equally grouped into two: GI: 24 animals (7 heifers and 17cows) and GII 24 animals (7 heifers and 17cows). Animals were induced for estrus using single 2ml intramuscular injection of prostaglandin (Synchromate B, Bremer pharma GMBH, GERMENY). Detection of estrus was performed twice daily (early morning and late afternoon) for 5 consecutive days of post PGF2α induction. Samples of straws from both groups were analyzed using conventional method for post-thaw motility before insemination. Animals were inseminated at standing heat (G-I with semen extended in BioXcell and G-II with semen extended in Tris egg yolk extender. Pregnancy was diagnosed in all animals on Day 28 post AI using Transrectal Ultrasonography.

Conception rate was defined as the number of animals that were confirmed pregnant after insemination and was computed as follows:

$$\text{Conception rate} = \frac{\text{Total conceived}}{\text{Total inseminated}} * 100$$

3.3.3. Rapid milk progesterone test

A total of 72 cows were included in this experiment. The test was applied on milk samples (Annex 1.) collected during milking on Days 18th and 22nd post insemination as described in the procedure included with the kit (Rapid P4, BIOMED, USA). The test result is confirmed from the stick, test and control lines. The presence of high progesterone is evidenced by absence of test line and appearance of only control line. If the level of progesterone is low both test and control lines are visible. Cows found positive on Day 18th for the test kit were repeated on 22nd day post AI for confirmation of the test result as described in the kit procedure. If the first test failed, the test was not repeated and the animal will be categorized as non pregnant.

3.3.4. Determination of serum progesterone concentration (Serum progesterone analysis)

The same animals (n=72) those have been sampled for rapid milk test were simultaneously sampled for serum. Blood samples were collected through jugular vein-puncture using a plain vacutainers on Days 18^{th} to 22^{th} post insemination. Serum was harvested and stored in cryovials at -20^{0C} degrees until analysis. Progesterone concentration was determined by EIA which is based on the principle of competitive binding between progesterone in the test specimen and progesterone-HRP conjugate for a constant amount of rabbit anti-progesterone. Serum P4 values <3ng/ml were considered as indicating non pregnancy and those ≥3ng/ml were taken as positive confirmation of pregnancy.

3.3.5. Transrectal ultrasonography

Animal previously sampled for progesterone analysis either in the milk or serum were examined on the date of sampling (Days 28^{th} to 30^{th} post AI) using a transrectal ultrasonography (B mode, linear array real time with 7.5 MHz frequency probe). The presence of anechoic area within the uterine structure or demonstration of fetal body part and a corpus luteum in the ovary were considered as a positive confirmation of pregnancy.

All cows confirmed positive with the two test procedures were subjected to transrectal palpation on Day 40 post AI for final confirmation of pregnancy maintenance. The sensitivity and specificity of the tests were computed based on the formula provided below. Computation of sensitivity and specificity of the tests was carried out as follows:-

Sensitivity (Se): was defined as the ability of the test to correctly detect pregnancy (true positive or probability of being positive when pregnancy is present).

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False Negative}}$$

Specificity (Sp): was defined as the ability of the test to correctly identify the non-pregnant animals (Probability of being test negative when pregnancy is absent).

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$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}$$

Gold standard (GS): is defined as the best test that is considered the current preferred method of diagnosing pregnancy. Accordingly, other tests were compared against findings of the GS.

3.4. Sampling Method

3.4.1. Milk sample collection and preparation for rapid test kit

Animals selected for sampling were restrained; write the ear tag (ID) number of the cow you are testing on the collection pot (tube). Before you collect the milk sample strip the teat three (3) times onto the floor. The milk sample collection for test needed from only one quarter. Always mix and shake well the milk to distribute the fat, with a clean pipette draw up some milk to the base of the bulb. Transfer the milk to the test tube to a maximum depth of 7mm. carefully, open the Rapid milk Progesterone pregnancy test kit packet at the notches and remove the stick. Write the ear tag (ID) number and date of cow on the white label. With the arrow pointing down, put the stick in the test tube and wait for five minutes for the result to show (the stick can stay in the milk longer if necessary), for ease of reading, let the stick dry then put it against a piece of white paper. The test result seen on the stick test and control lines.

The presence of high progesterone revealed on the test kit by absence of test line and only control line is displayed. If the level of progesterone is low both test and control line is seen clearly. The concentration of progesterone in the blood (serum) is correlated closely with the concentration in milk. In fact, since progesterone is a steroid hormone, it has an affinity for milk fat; thus progesterone in milk is somewhat higher than in blood. However, the relative relationship between milk and blood Progesterone levels is the same. Variation in the concentration of progesterone in milk due to stage of the cycle or pregnancy status is much greater than the effect caused by the variation in fat content. The blood sample is collected on the same day of milk test at days 18^{th} and 22^{nd} during Transrectal Ultrasonography scanning for serum progesterone concentration measurement.

3.4.2. Blood sample collection for serum concentration analysis

Blood samples were collected from post inseminated cows (n=72) from the jugular vein with the help of plain vacutainers tube with disposable vacutainers needles, for serum P4 concentration analysis on days 18^{th} and 22^{nd} post insemination.

The blood (10 ml) was collected and allowed to clot for 30 to 60 minutes at 15 to 20^{0C} and serum was separated by centrifuging the clotted blood at 3000 rpm for 15 minutes (Prakash *et.al.,* 1990). Serum was separated and stored at -20^{0C} deep freeze in plastic cryo vials. Serum Progesterone values <3ng/ml were considered non pregnant and ≥3ng/ml were taken as criteria for determination of pregnancy. Pregnant and non pregnant animals were diagnosed based on the serum progesterone values.

3.5. Data Management and Analysis

All collected data were entered, and organized in Microsoft excel sheet. Statistical analysis was conducted using SPSS software version 20. Logistic regression, X^2 and correlation for to see the association between two semen extenders conception rate, and early pregnancy diagnosis using rapid milk P4 test kit, Ultrasonography and Transrectal palpation. Percentage was used to estimate amount of animals conceived. In addition, a t-test was used to compare groups for post thaw evaluation of frozen semen extended with Bioxcell and TEY presented in mean (±S.E.M). The level of Statistical significance was observed at ($P<0.05$), and data was reported as percentage (%).

3.6. Ethical clearance (approval sheet)

Prior To Start of the Study Official Ethical Clearance For The Project Have Requested and Taken From Animal Research Ethics Committee of College of Veterinary Medicine and Agriculture, Addis Ababa University (Annex.2).

4. RESULT

Recorded values of fresh pre-freeze semen parameters viz mass motility [Score]; progressive forward motility [%], live percent [%] and morphological abnormality [%] were in the order of 2.89±0.13; 69.35±2.36%; 78.62±1.71% and 20.56±1.15%, respectively. Straws were packed at a concentration of 120mill/ml as per the standard of the National Artificial Insemination Center.

4.1. Post-thaw evaluation of frozen semen extended in Bioxcell and TEY

Post-thaw progressive motility in Tris egg yolk was significantly higher (43.59±3.77%; P<0.001) compared to that in BioXcell extender (40.83±4.01%; P<0.001). There was also a highly significant (P<0.001) positive correlation between progressive forward motility in fresh semen with that of post-thaw progressive forward motility both in Tris egg yolk (r=0.421) and in BioXcell extender (r=0.328).

Table 4: Descriptive results of viability and post-thaw evaluation of bull semen parameters in TEY and BioXcell extenders

	N	**Tris egg yolk extender**	**BioXcell extender**
Parameters		**Mean(±S.E.M)**	**Mean(±S.E.M)**
Viability after 1 hour [%]	24	67±2.67	68.11±2.61
Post-thaw progressive motility [%]	24	43.59±3.78	40.83±4.02
Post-thaw live percent [%]	24	47.22±1.85	46.52±1.73
Post-thaw total abnormality [%]	24	42.12±1.26	41.64±1.12

The live percent was significantly higher (P=0.000) in Tris egg yolk extended semen (47.22±1.85) as compared to the one extended in BioXcell (46.52±1.72). The correlation between live percent both in fresh semen and post-thaw samples preserved in TEY and BioXcell were significantly (p<0.001) higher (r=0.90; TEY) and in (r= 0.92, Bioxcell).

Table 5: Post-thaw morphological abnormalities in TEY and BioXcell extenders

Parameters		TEY	BIOXCELL
	N	Mean (±S.E.M)	Mean (± S.E.M)
Post-thaw head abnormality	24	6.77±0.34	6.41±0.30
Post-thaw mid-piece abnormality	24	4.62±0.32	4.79±0.32
Post-thaw tail abnormality	24	30.6±0.92	30.2±0.87

The total abnormality in TEY was significantly higher (42.1 ± 1.21; P<0.000) than that of BioXcell extended semen (41.64±1.12). Comparative figures of sperm abnormalities based on anatomical regions of the sperm are given in (Table 5).

Sperm reactivity to HOS test was higher in TEY extended semen than that of BioXcell extended semen (Table 6.). Live percent and progressive forward motility were correlated with HOS test reactivity only for Tris extended semen (r= 0.424 and r=0.331, respectively).

Table 6: Results of HOST reaction for bull semen extended in TEY and BioXcell extenders

Test	N	Mean (±S.E.M)	t-vale	P-value
HOST reactive sperm in TEY	24	8.87±0.72	12.316	0.000
HOST reactive sperm in Bioxcell	24	4.45±1.25	3.539	0.002

4.2. Conception Rate of semen extended in BioXcell and Tris egg yolk

First service conception rate was 50% for G-I and 70.8% for G-II. Though conception rate was higher in G-II, there was no statistically significant difference between the two groups (Table 7).

Table 7: First service conception rates from frozen semen extended in BioXcell and Tris egg yolk

Animals type	N	Extenders		PD tests
		Tris egg yolk	BioXcell	US
Heifer	14	71.42%	42.8%	57.1%
Cows	34	70.58%	52%	61.7%
Overall CR	48	70.9%	50%	60.4%
X^2		**9.049**		**11.782**
P value		**0.146**		**0.161**

Conception rate was not statistically significant (P>0.05) but higher for both heifers and cows in G-II (71.42%) and (70.58%) respectively.

4.3. Rapid Milk Progesterone Test (RMPT)

Out of the 72 cows tested for pregnancy, 42 cows (58.4%) were found to be positive with the RMPT. Sensitivity and specificity of the test were 88.9% and 72.2% respectively, taking Ultrasonography as a gold standard. From a total of 72 milk samples tested, negative results at the first test hence subsequent test was not carried out. Serum progesterone concentration diagnosed positive pregnancy in 37 (51.5%) of the cows and the average P4 concentration was 8.63 ± 3.93, ng/ml (Table 8).

Although progesterone detection was relatively higher with rapid milk test, there was no significant difference between the tests (Table 8). The accuracy of serum progesterone assay was 74.3% in diagnosing pregnancy and 86.4% for diagnosing non pregnancy. The Sensitivity and Specificity of determination of serum progesterone was 87.0% and 87.8%, respectively. Therefore, serum progesterone tests most accurate in determining non pregnancy, because the progesterone levels were below 1ng/ml at the test day.

Therefore, this test was most accurate in determining non pregnancy, because if the progesterone levels were not seen at test day the animal is considered as non pregnant and no further test performed. The rapid milk Progesterone tests showed that a sensitivity of 88.9% and a specificity of 72.2% and was the earliest proven method for identifying pregnant and non-pregnant animals early at days 18^{th} and 22^{nd} post breeding.

Table 8: Result of Rapid milk Progesterone test, Transrectal Ultrasonography, rectal palpation

Methods PD test	**Results of PD test**			
	Pregnant	**Non pregnant**	**False positive**	**False negative**
RMPT	58.3%	41.6%	**-**	**-**
US	44.4%	36.1%	13.8%	5.5%
RP	37.5%	62.5%	-	-
Sensitivity	88.9%	-	-	-
Specificity	-	72.2%	-	-
X^2		**11.782**		
P-value		**0.161**		

Key: *RMPT=Rapid milk progesterone test, US=Ultrasonography, RP= Rectal palpation, PD= pregnancy diagnosis*

4.4.Serum (Progesterone P4) Concentration Analysis

Serum progesterone analysis was done in 72 inseminated cows blood sample collected for serum on day 18^{th} and 22^{nd} post insemination. The concentration of serum P4 indicate either the animals conceive or the presence of reproductive abnormalities other than pregnancy. The level of progesterone on pregnancy and non pregnancy measure (>3ng/ml) and (<3ng/ml) respectively. Out of 72 tested for serum Progesterone concentration, 37/72(51.4%) were diagnosed as pregnant and 35/72(48.6%) as non pregnant and the mean values of serum progesterone concentration of pregnant and non pregnant were presented (Table 7). Out of pregnant cows diagnosed by this test, 32(86.5%) were confirmed pregnant and 5 (13.5%) as false positive or non pregnant upon transrectal Ultrasonography and transrectal palpation at days 28 - 30^{th} and 35-40^{th}. The test result in the present study, serum progesterone assay was 86.4% accurate in diagnosing pregnancy and 74.3% for non pregnancy. The test concluded that the determination of serum progesterone concentration at days 18th and 22nd post insemination tells the status of cows and the measurement of P4 concentration confirm the test predicted by rapid milk P4 test earlier at days 18th and 22nd post insemination.

Table 9: Results of Mean ±SE of serum progesterone concentration (ng/ml) 18th and 22nd days post insemination.

Diagnostic results	**Day 18th and 22nd post insemination**	
	No. of cow	**Serum P4 concentration**
Positive	37	8.63 ± 3.93ng/ml
Negative	35	1.272 ± 0.14ng/ml

Table 10: Early PD test by milk p4 and serum p4 concentration confirmed with transrectal Ultrasonography and rectal palpation

Diagnostic tests	**Diagnostic results**	
	Positive	**Negative**
Serum P4	37/72(51.4%)	35/72(48.6%)
US	32/72(44.4%)	26/72(36.1%)
RP	27/72(37.5%)	45/72(62.5%)

4.5.Transrectal Ultrasonography (US)

The test result of Transrectal Ultrasonography at day 28th was used as a gold standard test for confirmation of rapid milk Progesterone tested at day 18th and 22nd. A total of 72 cows were scanned using US for early pregnancy diagnosis on days 28-30th post insemination. Early detection of progesterone in milk at days 18th and 22nd indicate either the cow is pregnant or not, but this needs to be confirmed, because presence of progesterone between this days not only indicate the presence and absence of pregnancy, reproductive abnormality also detected us false positive. From examined 72 cows were 44.4% were found true positive, 36.1% was found true negative 13.8% was found false positive and 5.5% were found false negative. Transrectal Ultrasonography was used in different stages of gestations for scanning of early pregnancy and on this study used to evaluate the prediction of rapid Progesterone test accuracy. Transrectal Ultrasonography tells the sensitivity and specificity of the test result.

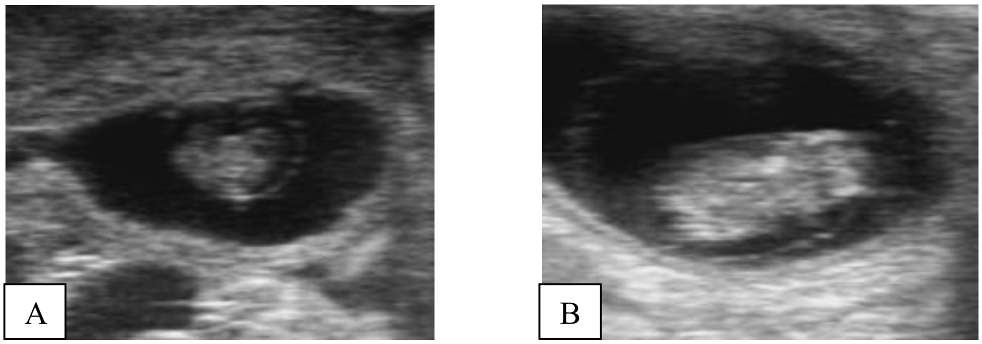

Figure 4: Transrectal Ultrasonographic images of early bovine embryos scanned at days 28 and 30 post inseminations
A= Ultrasound Image scanned at 28th day of pregnancy, B= Ultrasonic Image scanned at 30th day of pregnancy

Diagnostic tests	**N**	**Days post AI**	**Result**			
			Pregnant	**Non pregnant**	**False positive**	**False negative**
RMPT	72	18th to 22nd	42 (58.33%)	30(41.66%)	_	_
Serum P4	72	18th to 22nd	37 (51.4%)	35 (48.6%)	5(6.9%)	_
US	72	28th to 30th	32 (44.4%)	26 (36.1%)	10(13.8%)	4(5.55%)
RP	72	40th	27 (37.5%)	45 (62.5%)	15(20.8%)	_

The current study revealed that pregnancy detection was higher with RMPT. RMPT was the most sensitive but less specific taking Ultrasonography as a gold standard in detecting progesterone earlier, and better in detecting non pregnancy, Pregnancy later performed by transrectal palpation at day 40th post AI showed 27(37%) indicating the presence of early embryonic mortality. Serum progesterone assay intermediate in both sensitivity and specificity, Transrectal Ultrasonography is much better in both sensitivity and specificity of the tests and used as a gold standard test. The results false positive were due to the presence of persistent corpus leutem reproductive abnormalities, and false negative is miss interpretation of RMPT. RMPT was found to be easier to apply under field condition, and results comparable to Ultrasonography can be found if the test is repeated 7-8 days later. Diagnosis of pregnancy can also be made at an earlier stage using a RMPT under the field conditions of the most common production system in Ethiopia.

5. DISCUSSION

The study was aimed to evaluate conception rate of semen extended in Tris egg yolk and Bioxcell and to compare efficiency of early pregnancy detection methods using Ultrasonography and progesterone hormone analysis in dairy cattle. The results of the study have shown that, First service conception rate was 50% for G-I and 70.8% for G-II. Though conception rate was higher in G-II, there were statistically no significant differences between the groups. From a total of 72 animals inseminated for early pregnancy detection, pregnancy was confirmed on Days 18th and 22nd by RMPT and serum P4 analysis 42(58.4%) and 37(51.4%) respectively, and on Day 28th Ultrasonography 32 (44.5%) post AI. Although pregnancy detection was higher with RMPT, there was statistically no significant difference between the tests but RMPT was the most sensitive (88.9%) but less specific (72.2%) taking Ultrasonography as a gold standard.

Cryopreservation of sperm is of central importance to the artificial insemination industry. However, the cryopreservation of sperm is not something that nature anticipated. The cryopreservation and thawing of sperm has been shown to result in a loss of motility and membrane integrity and in a proportion of sperm, ends in cell death (Akhter *et al.,* 2010). The discovery of the protective effects of egg yolk and glycerol enabled sperm to survive the cryopreservation process and to preserve their fertility. The current study demonstrates that improved semen extenders for semen quality can be achieved via better semen extension medium under optimized conditions for better performance on preservation capacity and conception rate.

The homemade prepared Tris egg-yolk extender used for cryopreservation of bull semen yield better conception rate than commercially produced BioXcell. The result of this study was relatively comparative with previous reports on egg yolk based extenders (Fawad, 2013; Sul *et al.,* 2008; Bathgate *et al.,* 2006). Bull sperm cryopreserved in Tris egg yolk based yielded higher sperm motility and viability after thawing compared with commercially available extender as reported by (Celeghini *et al.,* 2008; Leite *et al.,* 2010). The current finding also agree partly with a report by (Berran *et al.,* 2012) who found better post-thaw motility, viability and longevity in semen extended with Tris egg yolk based extenders.

The cryo preservation of egg yolk-based extender was more effective and had better performance on at preserving total and progressive sperm motility and hence conception rate, which is in agreement with the present findings. The process of cryopreservation is known to cause damage to both the acrosome and sperm membrane leading to a premature capacitation and loss of fertilization ability (Lemma, 2010). The fact HOST reactivity was higher in Tris based extender in this study indicated its ability to prevent cryopreservation related sperm membrane damage thereby preserving sperm fertility.

However, contrary to the present finding Singh (2005*)* and Stradaioli *et al.,* (2007) reported a higher post-thaw motility in BioXcell preserved bull semen and it was argued that the higher content of glutathione in BioXcell as compared to Tris egg yolk based extenders might be the reason for the good post-thaw motility. Nevertheless, this single finding was not further substantiated by other studies from other researchers. In earlier studies, sperm cryopreserved in soybean- based extenders (BioXcell, Biociphos-Plus, Botu-Bov-Soy etc.) showed higher straightness and linearity when compared to Tris egg yolk extender, but a decrease on post-thaw sperm integrity was observed in BioXcell when compared to Tris egg yolk extender (Crespilho, *et al.,* 2012; Gil*, et al.,* 2000, and Vera, *et al.,* 2009).

These varying findings could have been due to differences in extender density, viscosity or even the presence of large particles in different extenders (Celeghini*, et al.,* 2008). Munoz *et al.,* (2009) suggested that BioXcell extender has less protective effect on maintaining spermatozoal plasma membrane integrity, and indirectly reflected on sperm motility, which was in agreement with our findings of breeding success of Tris egg yolk. (Akhter *et al.,* 2010) also found non-significantly higher values for plasma membrane integrity and acrosomal integrity of spermatozoa after thawing in semen frozen in Tris egg yolk than BioXcell, while our findings showed significantly better quality parameters and fertilization capacity semen frozen in Tris egg yolk than BioXcell extender.

The current finding also agree partly with a report by Berran *et al.,* (2012) who found better post-thaw motility, viability and longevity in semen extended with other egg yolk based extenders than Bioxcell. However, contrary to the present finding, Singh, 2005 and Stradaioli *et al.,* 2007 reported a higher post-thaw motility in Bioxcell preserved bull semen. It was argued that the higher content of glutathione in BioXcell as compared to Tris based extenders might be the reason for the good post-thaw motility in Bioxcell. Nevertheless, this single finding was not further substantiated by other studies from other researchers

The results of the present study revealed that frozen semen extenders compared on conception rate and ease of preparation at laboratory with cheaper coast shows better fertility performance. However, are contrary to the earlier report (Asr*,* *et al.,* 2011*),* wherein higher post-thaw motility, viability, acrosomal integrity and plasma membrane integrity of sperm were recorded immediately after thawing for insemination as well as after 6 h of incubation in Tris egg yolk extender than in standard BioXcell extender. Semen extenders can be locally prepared to mitigate the cost commercial extenders with even better conception results.

The present experimental study demonstrates the correlation of early pregnancy diagnosis using RMPT at days 18^{th} and 22^{nd} post insemination and Ultrasonography at 28 to 30 days post insemination. (Glatzel *et al.,* 2000; Muhammed *et al.,* 2000; Otava *et al.,* 2007) were reported results in agreement with the present study. Kaker *et al.,* (1993), Ropstad and Refsdal (1985) and Stevenson and Call (1986) reported sensitivity and specificity of 88.6% and 92.4%, RMPT

The accuracy of this rapid milk progesterone on detecting non pregnant cows observed in the present study was 89% and these were in agreement to the findings of (Singh and Puthiyandy 1980; Kaul and Prakash 2006; and Ucar *et al.* 2004; Inaudi *et al.,* 1988; Elmore 1986; Takeuchi *et al.,* 1997 reported 100 per cent accuracy. However, much lower result of 81.8% 79.6% 81% and 75%, respectively were reported by (Gupta *et.al*. 1991; Kaker *et al*., 1999; Wimpy *et al.,* 2007; and Nebel *et al*., 2003).

The pregnancy diagnosis by means of serum P4 levels in 28^{th} day post insemination was 75%. (Rajamahendran *et al.,* 1990; Naikoo *et al.,* 2013; Romagnolo & Nobel, 1993; Kaul and prakash, 1994; and Lec *et al.,* 1996), reported ELISA based serum progesterone kit detect pregnancy with

98% reliability on day 18^{th} – 24^{th} post insemination Based on Ultrasonography out of 72 cows scanned 44.4% were diagnosed as pregnant. Similar findings were reported by (Curran *et al.,* 1986; Pawshe *et al.,* 1994; Bhosrekar and Hungarge, 2000; Glatzel *et al.,* 2000), reported the visualization of embryonic vesicle early at day 28^{th} could give 100% pregnancy detection. Others include (Gupta *et al.,* 1991; Lackchawura *et al.,* 2003; Samad *et al.,* 2004; and Ucar *et al.,* 2004) reported relative similar values to present findings.

6. CONCLUSION AND RECOMMONDIUTIONS

Cryopreserving and fertilization capacity of sperm cell is dependent on extenders used for a better reproductive management. The result obtained after the experiments on extenders comparison, Tris egg yolk was found better conception rate than BioXcell. Tris based extender have the ability to prevent cryopreservation related dame to sperm membrane which is also one cause of loss of sperm fertility. Although all post thaw semen parameters are comparable, HOST reactivity indicates it supremacy over the commercially prepared extender. TEY extender also has the advantage of being locally prepared further allowing a wider use for Ethiopian condition.

Pregnancy can be diagnosed as early as 18^{th} – 22^{nd} day post AI while the methods have different level of sensitivity, the RMPT was much better in easiness of application and from cost perspective. Pregnancy test specificity is relatively better with serum P4 analysis, however, sampling is more invasive and the cost per samples is higher. Ultrasonography has the advantage of accurate pregnancy detection at an earlier stage however; its ease of application and cost of the machine makes it less preferable for field use. The results obtained after the experiment was performed, recommend the method described in this paper as a good practice to improve the reproductive management of dairy cattle farms by means of the early pregnancy detection, and increasing submission rate.

Early pregnancy diagnosis is an important aspect for optimizing dairy production, but unfortunately none of the present day methods qualifies as an ideal diagnostic easy to use mode due to limitations of accuracy, applicability and requirement for elaborate instrumentation and laboratory setup. In these cases, rapid tests are viable options for pregnancy diagnosis under field conditions at an earlier stage between days 18^{th} and 22^{nd} even by farm owners or attendants if they are trained. Based on the above conclusion the following recommendations are forwarded:

- An experiment on wider scale of the cryopreservation ability of tris based extender
- Making post-thaw evaluation of semen to be mandatory before AI

- Availing and adoption on the use and application of rapid pregnancy tests for the most common production system in Ethiopia
- Records should be kept after insemination or after a natural service with the possible identification of individual animals

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8. ANNEXES

Annex 1: Questionnaire for Animal owners to assess managemental practices of pregnant cattle

I. Questionnaire Number____________________

1. Identification

    1. Date of interview ________________ 2. Sex _______ 3. Age_______

    4. Kebele _________________ 5. Woreda _______________

2. Educational status

    1. Illiterate 2. Primary school

    3. Secondary School 4. Above Secondary School

3. Occupation:

    1. Farmer 2. Student 3. Employed 4.Other

II. Cattle herd structure

1. Do you have cattle? Yes______ No_______

2. Purpose of keeping cattle

    a. Traction b. Milk c. Both traction and milk d. Meat

    h. Other (specify) _________________

3. Do you have any crossbred cattle? Yes______ No_______

4. If yes, how did you get your crossbred cows?

    1. Purchased from neighbors 3. Purchased from market

    2. Purchased pregnant cow from any project 4. Through A.I

    5. Supplied by the MoA.

III. Cattle management, housing and breeding system

1. How you can manage your cattle

1.1. Housing system

    A. conventional barns housing system

    B. loose housing system

C. free range housing system

1.2. Feeding and availability of water

    A. concentrate

    B. hay and brewery end product

C. Ground water

2. What is your breeding system?

1. Natural breeding 2. Artificial breeding 3. Both

3. How you can identify cow/heifers for AI/ heat detection?

____

____

____

4. What is the time for insemination once you see the estrus signs?

____

____

5. What do you do if the AIT comes too late for insemination?

1. Get the service any way 2. Reject the service and wait for another 21 days

3). Use Natural Mating 4). Do not know

6. How you manage cow/heifers after insemination?

____

____

____

7. How you can identify whether cow/heifers pregnant or not?

____

____

____

8. How you handle/manage pregnant cow/heifers?

____

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Annex 2: Ethical approval sheet

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/04/08/11/2019

Name of Applicant: Tilaye Demissie (DVM, MSc, PhD fellow)

Address: College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *In vitro embryo production potential of Boran and Zebu-Holestein cross dairy cows in Ethiopia*

Date of application:	**13/11/2018**
Nature of the project:	**mildly invasive**
Target animal species:	dairy **cattle**
Number of animals involved:	40
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/08/11/018, 21/12/2018**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University

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Please quote Our Ref. No. When replying

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Annex 3: Pictures during works. AI, Rapid milk P4 test, US and RP

Key: A= VOG team on duty, B= Semen thawing C. Artificial Insemination of animals D. Transrectal Ultrasonography E. During Rapid milk progesterone test, F. RMPT result on the stick