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EVALUATION OF BIOCHEMICAL DETERMINANTS OF SUCCESSFUL CONCEPTION IN DAIRY COWS IN AND AROUND BISHOFTU, ETHIOPIA

MVSc THESIS

BY

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DEPARTMENT OF CLINICAL STUDIES

MVSc IN VETERINARY OBSTETRICS AND GYNECOLOGY

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BISHOFTU, ETHIOPIA

EVALUATION OF BIOCHEMICAL DETERMINANTS OF SUCCESSFUL CONCEPTION IN DAIRY COWS IN AND AROUND BISHOFTU, ETHIOPIA

A Thesis Submitted to College of Veterinary Medicine and Agriculture of Addis Ababa University in Partial Fulfillment of the Requirements for Degree of Masters of Science in Veterinary Obstetrics and Gynecology

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As members of the Examining Board of the final MVSc open defense examination, we certify that we have read and evaluated the Thesis prepared by: Kutelo Kussia, Title: **Evaluation of Biochemical Determinants of Successful Conception in Dairy Cows in and around Bishoftu, Ethiopia** 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 mother, Ayana Adale, my father, Kussia Muchole, my wife, Getenesh Geyola, and my friends for nursing me with affection and love and for their dedicated partnership in the success of my life.

STATEMENT OF THE 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 (MVSc) 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 thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

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BIBLIOGRAPHY

Kutelo was born in 1987 from his father Kussia Muchole and his mother Ayana Adale in Fasha, a small kebele in Kena District, Konso Zone, southern Ethiopia. He attended primary school in Fasha primary school starting in 1995 and attended his high school in Fasha secondary and preparatory school until 2007. After he completed high school, he joined Wolaita Sodo University and has been attending veterinary medicine since 2008. After graduation, he worked at Wolaita Sodo University in southern Ethiopia as a lecturer for one year. He again joined Addis Ababa University, College of Veterinary Medicine and Agriculture to study his master of veterinary science in veterinary obstetrics and gynecology from September 2015 to date.

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
BCS	Body Condition Score
CM	Cervical Mucus
CR	Conception Rate
CRS	Crystallization
CSA	Central Statistical Agency
CSU	Colorado State University
DM	Dry Matter
DMI	Dry Matter Intake
E2	Estrogen
ECW	Extra Cellular Water
FMD	Foot and Mouth Disease
LH	Luteinizing Hormone
NEB	Negative Energy Balance
P4	Progesterone
PGF2α	Prostaglandin F2α
PMN	Polymorphonuclear Neutrophils
RB	Repeat Breeding
SBK	Spinnbarkeit
SP	Sperm Penetration
VER	Vaginal Electrical Resistance

ABSTRACT

The influence of biochemical characteristics of cervical mucus at the time of estrus were evaluated on conception rates of normal-cycling and repeat-breeding dairy cows. A total of 62 Holstein-Friesian dairy cows, 38 normal-cycling and 24 repeat-breeding cows, were included in the study. Vaginal electrical resistance (VER) was measured before artificial insemination and cows were categorized into three VER groups (150-180, 181-220 and >220 Ω). The cows were also grouped into three body weight ranges (300-350, 351-400 and 401-450 kg). Cervical fluid parameters including pH, spinnbarkeit, sperm penetration and crystallization patterns were measured or scored and pregnancy was diagnosed by ultrasonography after 30 days post-AI. The overall conception rate was 54.84% with a significant difference ($P<0.05$) between normal cycling cows (63.2%) and repeated breeders (41.7%). The mean (±SD) VER was in the order of 219.03±66.17Ω and 221.67±41.77Ω for normal-cycling and repeated breeding cows, respectively. The average pH and spinnbarkeit value of cervical fluid were 7.38±0.32, 5.76±2.2cm in normal cycling and 7.32±0.27, 5.85±3.77cm in repeat breeder cows, respectively. The mean(±SD) VER of pregnant and nonpregnant cows were 202.15±31.15Ω and 241.79±73.56Ω, respectively. Body weight, timing of insemination and VER showed significant effects ($P<0.05$). Conception rates were highest for cows weighing 351- 400 kg(75%), inseminated 7-12 hours (71.43%) after standing estrus and VER of 181-220 Ω (84.21%). Interestingly, although there was some tendency for influence of the cervical fluid pH, this was not statistically significant, but sperm penetration, spinnbarkeit value and crystallization patterns of cervical fluid did ($P<0.05$). Cows with normal sperm penetration (72.2%), spinnbarkeit of 7-9 cm (87.5%) and typical crystallization patterns of cervical mucus (76.47%) had comparatively higher conception rates. In conclusion, sperm penetration, spinnbarkeit, vaginal electrical resistance and crystallization pattern are useful predictors of optimal insemination timing in Holstein cows.

Key word: *Dairy cows, Cervical fluid, Conception rate, Crystallization pattern, Sperm penetration, Vaginal electrical resistance*

1. INTRODUCTION

Ethiopia has great potential for dairy improvement with its abundant livestock and favorable climate for high-yielding cattle breeds (Ahmed *et al*., 2004). Despite this, the majority of dairy animals are low-yielding local breeds. Reproductive efficiency is crucial for dairy production, aiming for prompt pregnancies after calving (Plazier *et al*., 2002). However, reproductive success is hindered by factors like anestrus, genetics, congenital factors, infertility, hormonal issues and mismanagement such as inadequate nutrition and poor estrus cycle detection in dairy herds (Chakravarthi and Balaji, 2010).

Fertility plays a crucial role in a dairy cow's overall performance, with successful reproduction relying on the production of healthy sperm and eggs, as well as maintaining optimal conditions within the female genital tract for fertilization, implantation and fetal development. Effective heat detection, proper semen handling, precise insemination timing and a conducive uterine environment are all critical factors in ensuring the cow's ability to conceive (Alemneh *et al*., 2015). However, a significant challenge in achieving successful conception lies in the accurate detection of cows in estrus, which can lead to repeat breeding due to intrinsic reproductive issues or errors in identifying the cow's fertile period (Diskin and Kenny, 2014; Barui *et al*., 2015).

Repeat breeding in cows, defined as failure to conceive after three or more consecutive inseminations despite showing clear estrous signs without detectable reproductive disorders, can be attributed to hormonal asynchrony affecting cervical mucus properties, leading to reduced fertility (Perez-Marin and Quintela, 2023). Studies indicate that around 25% of repeat-breeder cows experience fertilization failure (Islam *et al*., 2023). Efficient estrus detection is crucial for successful conception, with techniques like visual observation, changes in vaginal mucus resistance and recording mounting activity playing key roles in effective breeding programs (Eshete *et al*., 2023). Accurate estrus detection is vital for addressing the challenges of repeat breeding and enhancing reproductive outcomes in dairy cows (Layek *et al*., 2011).

Over years, advancements in electronic technologies have greatly improved the efficiency of detecting estrus in cattle and sows. Research indicates that measuring electrical resistance near the cervix uteri is a useful method for identifying estrus and ovulation, helping to determine the best time for artificial insemination in both animals (Gunduz and Basciftci, 2023; Sasankar *et al.*, 2023). Furthermore, the resistivity of vaginal mucus changes with the biochemical composition during different estrous cycle stages, offering valuable information for accurate estrus detection (Tsiligianni *et al*., 2001).

The characteristics of cervical mucus have a significant impact on sperm activity in the female reproductive tract (Lim *et al*., 2014). These characteristics are crucial in conception and essential for natural fertilization and sperm capacitation (Maher *et al*., 2018). Cervical mucus also serves as a biological environment that influences sperm survival and affects the ability of cows to conceive (Beran *et al*., 2013). Key biochemical features of cervical mucus including pH, spinnbarkeit, crystallization and sperm penetration are valuable laboratory indicators for predicting infertility in cattle due to their significant influence on the sperm's fertilizing capacity (Rangnekar *et al*., 2002).

Analyzing fluids produced in the female reproductive system to determine fertility has become a significant focus. Cervical fluid plays a role in sperm survival and transport to the uterus (Kumar *et al*., 2012). The characteristics of cervical mucus can be used to pinpoint the precise timing of artificial insemination and serve as an indicator of reproductive health related to estrous behavior. Moreover, cervical mucus quality significantly influences the success of pregnancy, consequently enhancing the rate of conception (Siregar *et al*., 2019; Abd-El-Hafeez *et al*., 2020).

Studying fluids from cows, specifically cervical mucus, is essential for comprehending reproduction. According to Biradar *et al*. (2016), electrolytes in cervical mucus are crucial for supporting sperm viability and transport. Research on biochemicals in the reproductive system has mainly focused on women, with limited published studies in cows (Simonik *et al*., 1991).

1.1. Statement of the Problem

Failure of a cow to become pregnant or maintain pregnancy is an important variable that accounts for a large percentage of reduced reproductive efficiency. There are a variety of factors that can influence conception rates in cows. The majority of the variation in conception rates can be attributed to environmental factors, management factors and genetic factors of cows and service bulls. Differences in herd nutrition, metabolic disorders, reproductive health, heat detection, insemination practices and climate can significantly impact conception rates (Kathy Lee, 2004). One of the unchartered areas is the role of the biochemical characteristics of the female genital tract at the time of estrus.

1.2. Hypothesis of the Study

A repeated breeder dairy cows have a less favorable uterus environment for sperm, so after insemination, sperm dies immediately and pregnancy fails. Sperm interactions within the female genital tract between normal cycling and repeated breeder dairy cows are the basis for the difference in ability to conceive, therefore, this appears to be the problem of conception after insemination.

Objectives of the present study were:-

- To study the physico-biochemical properties of utero-vaginal secretion in dairy cows during the estrus period in relation to fertility.
- To evaluate the role of biochemical characteristics of the cervical mucus at the time of estrus on conception rate in dairy cows.
- To evaluate the differences in physico-biochemical properties of cervical mucus between normal cycling and repeat breeders dairy cows.

2. LITERATURE REVIEW

2.1. Microscopic Anatomy and Physiology of the Genital Tract in Cows

The genital tract of cows consists of various structures, including the vulva, vagina, cervix, uterus, oviducts and ovaries. These structures undergo dynamic changes throughout the estrus cycle, which is typically 21 days in duration. The estrus cycle is primarily driven by fluctuations in the levels of reproductive hormones, such as estrogen and progesterone (Noakes *et al*., 2009). The vaginal epithelium, which lines the vaginal wall, undergoes significant changes during the estrus cycle. During the proestrus and estrus phases, when estrogen levels are high, the vaginal epithelial cells proliferate and become more keratinized, leading to the production of a thin, watery and clear vaginal discharge (Hafez and Hafez, 2000).

Alterations in vaginal mucosa cell cytology were observed due to fluctuations in hormonal levels during ovarian estrous cycles. During the estrous phase, there was a high concentration of estrogen in the bloodstream, leading to heightened uterine activity, increased secretion, keratinization of vaginal epithelial cells and subsequent proliferation of epithelial cells through elevated mitotic cell division (Popalayah and Ngadiyono, 2013).

The lining of the cervix, known as the cervical mucosa, undergoes changes in response to hormonal shifts. When estrogen levels are high during the proestrus and estrus phases, the cervical glands produce a thin, watery mucus that aids in sperm transportation (Hafez and Hafez, 2000). This mucus, which is high in water content and low in viscosity, facilitates sperm penetration. These changes in the vaginal and cervical tissues are essential for successful breeding in cows. Hormonal regulation plays a critical role in creating optimal conditions for sperm transport, fertilization and pregnancy establishment (Noakes *et al*., 2009).

Hormones are crucial in affecting cervical cell secretion in cattle. The levels of estrogen and progesterone during the estrous cycle impact the composition and characteristics of bovine cervical mucus. Higher levels of estrogen result in increased mucus production, which becomes more watery and less sticky, aiding in sperm transportation. Furthermore, estrogen encourages mucus crystallization, leading to fern-like patterns, especially during estrus (Verma *et al*., 2014). Understanding these hormonal effects on cervical mucus secretion is essential for assessing the fertility status of dairy animals and determining the best timing for insemination in cattle (Breeveld-Dwarkasing *et al*.,2002).

2.2. Estrus Signs in Dairy Cows and its Detection Methods

Dairy cows are polyestrous animals, meaning they experience estrus regularly throughout the year. The estrous cycle, which is the time between estrous periods, typically lasts 18 to 24 days in bovines, with an average duration of 21 days (Diskin and Sreenan, 2000). In dairy cattle, estrus, also known as being in heat, lasts 12 to 18 hours and ovulation usually happens towards the end of this period or within 6 to 10 hours thereafter (DuPonte, 2007). Throughout the estrous cycle in dairy cows, there are cyclic changes in the morphology of their reproductive organs and behavior. The decrease in progesterone (P4) concentrations from 5 – 7 ng/mL to less than 2 ng/mL during luteolysis at day 18 to 19 marks the regression of the corpus luteum (Sartori *et al*., 2004).

Various tools are available to help producers detect estrus in their cattle, such as pressure mount detectors, tail paint, androgenized cows and teaser bulls. However, the heat watch electronic system has been found to be the most effective in providing accurate information on the timing, strength and duration of estrus (Rorie *et al*., 2002). Additionally, estrus can also be detected through examining cervical mucus during the cycle, especially around the peak of estrus (Silaban *et al*., 2012). The most common method for detecting estrus is visually observing changes in cow behavior, particularly standing heat. Studies have shown that the efficiency of visually detecting estrus through standing heat can vary widely, ranging from 90% to less than 50% (Van Eerdenburg *et al*., 2002; Roelofs *et al*., 2006).

Estrus detection in cattle involves observing various behavioral and physical signs indicative of the fertile period. The primary and most definitive sign is the female standing to be mounted by a bull or herd mate (Forde *et al*., 2011; Graves, 2012; Fricke, 2015). Secondary signs include a roughened tail-head, nervousness, restlessness, riding or mounting other animals, grouping behavior, clear mucus discharge, swollen and red vulva, chin resting and rubbing, frequent urination, bawling, sniffing, decreased milk production and reduced feed intake. These signs collectively provide valuable cues for determining the optimal breeding timing in cattle (Duponte, 2007; Graves, 2012).

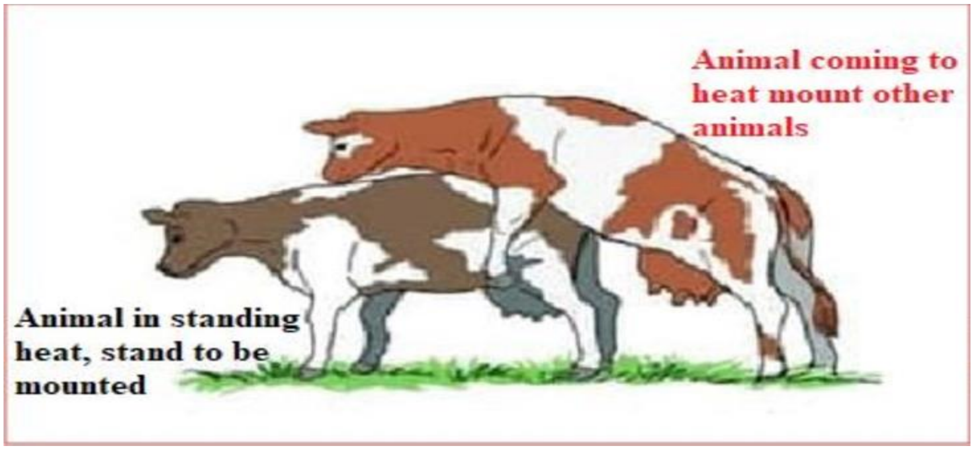

Figure 1. Schematic representation of a cow in heat and coming to heat

Source: (Roelofs *et al*.,2005).

Besides the behavioral indicators, researchers have explored various nonbehavioral methods for identifying estrus. Nonvisual signs, which are less subjective, enable the use of remote sensing and automation. Changes in the progesterone level in milk, vaginal electrical resistance, chemical composition of vaginal mucus, body temperature and pH of vaginal mucus constitute some of the nonvisual indicators of fertility (Saint-Dizier and Chastant-Maillard, 2012).

Vaginal mucus technology using electronic resistance is a helpful tool for detecting estrus in farm animals. Estrus, characterized by increased estrogen levels, can impact metabolic pathways and lead to the production of hormones like adrenocorticotropic hormone and aldosterone (Noakes *et al*., 2019). This can elevate the sodium chloride levels in the vaginal mucus. Research has shown that vaginal electrical resistance decreases during estrus, with the lowest readings aligning with the best time for insemination (Hockey *et al*., 2010). The decline in electrical resistance is most noticeable during estrus, with cows showing higher pregnancy rates when inseminated during low electrical resistance periods compared to high electrical resistance periods (Ahmed *et al*., 2017).

Measurements of alterations in electrical resistance of vaginal and cervical mucus, with varying degrees of efficacy. Variability in electrical resistance during estrus has been demonstrated both within animals across successive estruses and among animals during the same day of the estrous cycle in cattle. Estrus in a cow is indicated by low resistance readings (30 to 40 ohms); however, not all animals exhibit low readings during estrus (Rorie *et al*.,2002). Factors like the placement of the probe in the vagina, presence of infection, and animal temperament can lead to inaccurate or irregular resistance readings (Vinas *et al*., 2022).

The vaginal pH drops significantly one day before estrus begins and continues to decrease at the start of estrus, reaching its lowest point just before ovulation (Utomo *et al*.,2023). Ganesan and Kadalmani (2016) also noted a pH drop during estrus, with the lowest pH recorded four to six hours after estrus onset. However, this method has limitations due to pH probe placement variability and differences between cows. Reproduction management is crucial for the economic success of dairy farms, with estrus detection playing a key role in successful conception (Layek *et al*., 2011).

A significant challenge for optimal reproductive performance in farm animals is the difficulty in detecting estrus. This challenge is primarily due to environmental factors such as extreme temperatures, housing conditions and changes in nutrition, as well as physiological factors like silent estrus, anestrus, short estrus periods and estrus onset during nighttime (Felton *et al*., 2012). Failure to detect cows in estrus is a key factor contributing to reduced fertility (Law *et al*., 2009). Incorrect or inadequate estrus detection can lead to cows becoming repeat breeders (Rorie *et al*., 2002) and mistakenly categorized as anestrus. Variations in the female genital system, both physical and chemical, play a crucial role in determining the optimal timing for insemination. While different farmers use different methods of estrus detection and hence have differences in the determination of the optimal time for AI, there is a universally accepted schedule for optimal breeding time (**Figure 2**).

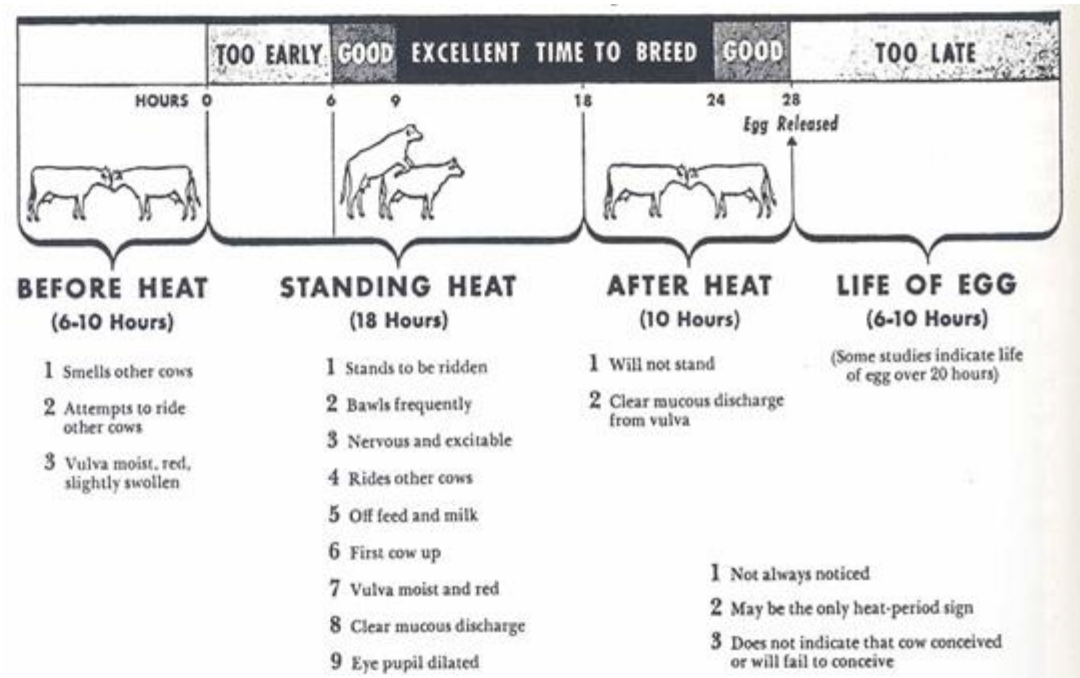

Figure 2. Timing guide for an average cow; Source: (Mikel, 2009)

Estrus detection plays a crucial role in enhancing the production and reproductive efficiency of dairy cattle, particularly in farms that heavily rely on artificial insemination techniques (Reith and Hoy, 2018). Regular monitoring of estrus activity in cattle is essential, with recommended observations at least twice daily. Timing is key, as cows showing signs of estrus in the morning should undergo insemination later that same day, while those exhibiting heat in the afternoon should be bred the following morning (Graves, 2012). This timely detection and management of estrus greatly contribute to successful reproductive outcomes in dairy cattle.

2.3. Biochemical Changes of the Genital Tract during the Estrus Cycle

2.3.1. Vulva

The vulva of cows undergoes distinct biochemical alterations throughout the estrous cycle. As cows approach estrus, the vulva exhibits signs of redness, swelling, and clear mucus discharge, which were associated with high estrogen levels. The increase in estrogen levels causes changes in the rheological properties of the cervical mucus, facilitating the passage of spermatozoa (Laksmi and Trilaksana, 2020).

The swelling of the vulva during estrus is caused by a movement of water from inside the cells to the outside (Ranasinghe *et al*., 2021). The changes in cellular water content from inside to outside as the cow approaches estrus are gradual as circulating estrogens in the blood increase, and the reverse is true; circulating estrogens post-estrus decline and progesterone levels increase, causing the cellular water to diffuse back into the cell (Reith and Hoy, 2018). The increase in extracellular water (ECW) is primarily responsible for the increase in total tissue water during estrus (Bianchi *et al*., 2020). These dynamic shifts in fluid balance within the vulvar tissues contribute to the characteristic edematous appearance observed clinically around the time of estrus in cattle (Sa Filho *et al*., 2022).

The research conducted by various authors sheds light on the dynamic changes in ion concentrations, particularly potassium (K+), sodium (Na+), and chloride (Cl-), in the reproductive tissues of female cows during different phases of the estrous cycle. Studies indicate that during estrus, potassium levels are notably low in the endometrium, reflecting significant fluctuations in ion concentrations in the vulvar tissues (Maksymyuk *et al*., 2022). Furthermore, the increase in extracellular water content during estrus is linked to alterations in tissue sodium and chloride levels, highlighting a complex interplay of ion concentrations throughout various reproductive phases (Salleh *et al*., 2022). These findings underscore the intricate relationship between ion dynamics and reproductive physiology in cows, emphasizing the importance of understanding these changes for fertility and reproductive success (Bernardi e*t al*., 2018).

2.3.2. Vagina

The vaginal mucosa of cows exhibits distinct biochemical changes throughout the estrous cycle. The decrease in vaginal pH is accompanied by changes in the accumulation of ions, such as hydrogen, sodium and chloride, as well as alterations in the levels of glycogen and protein in the vaginal tissues. These dynamic shifts in the biochemical composition of the vaginal environment facilitate the transport and survival of spermatozoa, as well as the fertilization process around the time of ovulation (Agbugba *et al*., 2020).

The pH level of the vagina decreases during ovulation, as observed by Khalifa *et al*. (2010). Towards the end of estrous, Rezac *et al*. (2001) noted that vaginal mucus becomes more alkaline. This shift in pH is influenced by the presence of ions such as hydrogen, sodium and chloride in the vaginal environment and is also associated with the accumulation of glycogen and protein (Khadiga *et al*., 2005). Additionally, Blaszczyk *et al*. (2004) highlighted that the decrease in vaginal pH correlates with the release of LH during ovulation. During estrus, the vaginal surface epithelial cells undergo significant expansion due to mucus intake, leading to maximal height. The number of cell layers in the surface epithelium varies across different vaginal regions, ranging from one to five layers of polyhedral or squamous cells arranged in clusters near the propria membrane. Within a day post-estrus, the structure of the surface epithelium closely resembles that observed during estrus (Noakes *et al*., 2018).

In the initial days following estrus, the surface epithelium undergoes a reduction in height and adopts a cuboidal shape, with irregularities appearing on the luminal cell edges. During this stage, the squamous cell layers reach their peak. Subsequently, three days post-estrus, the superficial epithelial cells transition from columnar to cuboidal for the remainder of the estrus period. As estrus progresses, the squamous cells diminish gradually and are minimal by the end of estrus. The period of proestrus sees an increase in the depth of surface epithelial cells, with a transformation in cell shape from cuboidal to columnar (Noakes *et al*., 2018).

Vaginal mucus, originating from secretory cells in the endocervix within the cervical region of the genital tract, exhibits varying resistivity levels influenced by the changing composition of its biochemical constituents during different stages of the estrous cycle (Tsiligianni *et al*., 2001). During estrus, mucus production intensifies to facilitate the transportation of sperm to the site of fertilization. This augmented secretion is primarily driven by elevated estrogen levels and decreased progesterone levels. The upsurge in estrogen levels prompts the secretory cells in the oviduct, uterus, cervix, and vagina to enhance mucous production. These secretions amass in the vaginal environment, intermixing with leukocytes and cellular remnants from the oviduct, uterus, cervix, and vaginal epithelia (Hafez, 1987).

Vaginal mucus possesses distinct rheologic characteristics, including flow elasticity, viscosity, and ferning, all of which exhibit cyclic patterns. The cyclic fluctuations in mucus viscosity lead to periodic alterations in spermatozoa permeability within the cervical canal (Bernardi *et al*., 2016). These changes in mucus composition during estrus are crucial for sperm penetration and ovum fertilization. The ferning pattern, attributed to the mucus's elevated chloride levels, is absent in mucus samples collected during high progesterone levels or pregnancy (Cortes *et al*., 2014).

During the estrous cycle of female domestic animals, changes in vaginal mucus composition occur, whereby during ovulation, the mucus becomes abundant, thin, elastic and sticky, while in the post-ovulatory phase, it transitions to a thick and clumpy consistency cheesy (Nasirud-Din *et al*., 2003; Mahmoud, 2009). These alterations in the physical properties of vaginal mucus have been long recognized as an indicator of fertility in cycling mammals (Nasir-ud-Din *et al*., 2003). For instance, in Holstein cows, observations of the visual appearance (Bernardi *et al*., 2016), viscosity and crystallization patterns of vaginal mucus have been utilized to determine the optimal timing for insemination (Tsiligianni *et al*., 2000).

During estrus, elevated estrogen levels are believed to enhance metabolic pathways responsible for the production of adrenocorticotropic hormone and aldosterone. These hormones contribute to an increase in sodium chloride concentration in vaginal mucus. Sodium chloride acts as an electrolyte when dissolved in water, reducing electrical resistance and allowing for greater conductivity. Consequently, high estrogen levels lead to elevated sodium chloride levels, resulting in decreased electrical resistance and lower Draminski readings (Fehring,1997).

In the biochemical composition of vaginal mucus, elevated levels of electrolytes such as sodium, magnesium, calcium, and chloride can induce greater ionization, leading to the decreased electrical resistivity of the mucus during estrus (Agbugba *et al*., 2020; Mohamed *et al*., 2022). These electrolytes, in conjunction with other biochemical factors such as pH, total protein, cholesterol and potassium, demonstrate notable fluctuations throughout the various phases of the estrous cycle, contributing to the estimation and categorization of estrus .

2.3.3. Cervix

The cervix is a fibrous organ that separates the uterus and the vagina. The cervix is composed mainly of connective tissue, mucus-secreting cells, ciliated cells and small amounts of smooth muscle. The connective tissue of the cervix is made of ground substances, fibrous constituents and cellular elements (Hafez and Hafez, 2000). The uterine cervix has many Nabothian glands that secrete mucus. The cervical mucus consists of macromolecules of mucin of epithelial origin which is composed of glycoproteins (such as lipoproteins and albumin), carbohydrates (such as galactose, glucosamine and sialic acid) and enzymes (glucuronidase, amylase, phosphorylase, esterase and phosphatase). Cervical mucus has many rheologic properties (elasticity, viscosity and stickiness). The estrual cervical mucus shows a fern pattern on drying on a glass slide. This appears due to the high chloride content of the mucus (Cortes *et al*., 2014).

In the estrus stage, cervical mucus, produced by the cervix uterus under the influence of estrogen, adheres to specific areas of the vulva (Abidin *et al*., 2012). Estrogen, originating from ovarian follicles in estrus animals, plays a crucial role in enhancing the secretion of cervical mucus and regulating estrus intensity (Ismail, 2009). During heat and ovulation, congestion and edema occur in the cervix, facilitating its gradual relaxation and dilation (Lim *et al*., 2014). Following heat, the cervical mucus-secreting cells undergo morphological changes, becoming cuboidal in shape with oval nuclei positioned less basally.

The cervical mucus, a viscoelastic secretion generated by mucus-secreting epithelial cells in the cervix, acts as a crucial factor in natural fertilization by influencing sperm survival and capacitation (Maher *et al*., 2018). It plays a pivotal role in determining the fertility of cows through its impact on sperm survival and migration (Beran *et al*., 2013). Additionally, cervical mucus functions as a chemical and physical barrier that regulates the movement of sperm cells and offers protection against microbial invasion (Verma *et al*., 2014).

Cervical mucus, primarily composed of water, ions, enzymes, mucins, plasma proteins and bactericidal proteins (Martyn *et al*., 2014), is regulated by reproductive hormones (Siregar *et al*., 2019). Variations in hormone levels influence the quantity, constituents and structural characteristics of cervical mucus during the estrus cycle, impacting sperm permeability and potentially leading to fertilization challenges (Nakano *et al*., 2015). An elevation in the concentration of water and electrolytes within cervical mucus induces changes in the glycoprotein-to-water ratio, consequently modifying its rheological characteristics. This results in an increase in the water content, making the mucus more translucent, less viscous and easier for spermatozoa to navigate during the follicular phase in comparison to the luteal phase of the oestrus cycle. During the luteal phase, the mucus is sparse, highly viscous and poses challenges to sperm penetration (Rutllant *et al*., 2005).

In female reproductive tracts, the quality of cervical mucus, influenced by semen depositional capacity, is crucial for fertility status (Rangnekar *et al*., 2002). During estrus, cattle expel clean cervical mucus of high quality, emanating from the cervix without odor, which is associated with increased conception rates during artificial insemination (Kuswati *et al*., 2022). Conversely, irregular cervical mucus can hinder reproductive performance (Mahmoudzadeh *et al*., 2001), affecting sperm endurance and transport to the uterine cavity (Kumar *et al*., 2012). Alterations in cervical mucus composition serve as indicators for determining the optimal timing for artificial insemination (Tsiligianni *et al*., 2000).

The key physical attributes of cervical mucus crucial for fertility include pH, spinnbarkeit value, crystallization, penetration ability and viscosity (Tsiligianni *et al*., 2001). These characteristics are linked to the E2 / P4 ratio and fertility status of dairy animals (Rangnekar *et al*., 2002).

Crystallization plays a significant role in sperm penetration through the cervix (Tsiligianni *et al*., 2001; Rutllant *et al*., 2005), with ferning being the result of crystallization leading to the formation of a fern leaf-like structure in the mucus due to minerals such as calcium, zinc, magnesium, potassium and sodium. Higher salt concentrations are associated with more pronounced ferning patterns (Savia *et al*., 2021).

One important factor influencing successful pregnancy in livestock is the pH level of cervical mucus, which serves as a crucial transport medium for sperm. Research has shown significant variations in pH values of bovine cervical mucus, which play a key role in creating an optimal environment for both sperm and ovum during the reproductive process (Siregar *et al*., 2019).

In bovine cervical mucus, various elements like potassium, magnesium, calcium, copper, zinc, chromium, cadmium, mercury, phosphates and bicarbonates are present, similar to uterine and vaginal fluids (Tsiligianni *et al*., 2002). While most research on these elements has focused on women, limited studies have been conducted on cows. Among these elements, chromium, cadmium, mercury and zinc have been detected in cow cervical mucus, with zinc being associated with reduced conception rates (Simonik *et al*., 1991).

2.3.4. Uterus

In cows, the histological and morphological changes in the endometrium during the estrous cycle are closely tied to the influence of ovarian hormones and the activities of the pituitary gland (Yevtukh, 2022). These alterations, occurring before and after ovulation, are crucial for preparing the endometrium to support implantation and nourishment of the fertilized egg. During estrus, the epithelial cell nuclei in the uterine lining and gland ducts become elongated and basally positioned, while the glandular lumina are small due to the tall epithelial cells (Ponomarjova *et al*., 2020). Leucocytes are consistently present in both the stroma and epithelium throughout the cycle, with the highest concentrations observed during proestrus and estrus (Bondarenko *et al*., 2019).

During the luteal phase of the estrous cycle, the endometrium undergoes significant changes, including increased glandular growth and branching. The cytobrushing technique is recommended for obtaining endometrial cytological samples due to its ease, speed, safety and effectiveness (Kasimanickam *et al*., 2005; Barlund *et al*., 2008; Oral *et al*., 2009). Cows experience various physiological and structural changes throughout the estrous cycle, such as heightened infiltration of polymorphonuclear cells (PMN) into the endometrium, particularly from proestrus to metestrus (Ohtani *et al*., 1993).

The concentrations of sodium, potassium, calcium, and chloride in the uterus of cows vary throughout the estrous cycle, with differences observed between species. Research on bovines and water buffaloes indicates that during estrus, lower levels of uterine fluid calcium are present compared to serum levels. Higher levels of calcium are found in pro-estrus and estrus stages compared to metestrus and diestrus. Additionally, the magnesium content in uterine fluid is consistently higher than serum levels except during estrus (Alavi-Shoushtari *et al*., 2012).

2.4. Survival of Spermatozoa in Female Reproductive Tract

Physiochemical and immunologic factors present in the vagina and cervix during insemination are critical for the survival and transportation of sperm to the uterus and oviduct. Vaginal secretions have been shown to immobilize sperm within 1 to 2 hours post-insemination, emphasizing the importance of swift transport to a more conducive environment (Hafez and Hafez, 2000). These secretions, containing electrolytes such as sodium, magnesium, calcium, and other cations in addition to essential substances for sperm survival, play a significant role in determining the electrochemical properties of the vaginal mucus (Ahmed *et al*., 2017).

Spermatozoa undergo capacitation and acrosome reactions in synchronized sequences influenced by different microenvironments in the cervix, endometrium, and oviduct, ensuring survival and migration for fusion with the oocyte (Vigil *et al.*, 2015*).* Additionally, the cervical mucus plays a crucial role in sperm passage, with its properties varying throughout the estrous cycle, becoming more penetrable around ovulation and being secreted by glands that direct spermatozoa towards the cervical canal walls.

The female reproductive system exhibits varying pH levels and conductivity levels in different regions. The alkaline conditions and high electro-conductivity in the genital tract enhance sperm motility but may decrease sperm survival, as noted by Yilma (2020). Conversely, the cervix provides a more hospitable environment for sperm survival due to its slightly acidic pH and lack of leukocytes, allowing spermatozoa to remain viable for up to 3 days, as discussed by Yahia *et al*. (2020). This favorable environment in the cervix plays a crucial role in maintaining a continuous supply of spermatozoa in cows' uterine horns, ultimately supporting successful fertilization and reproductive outcomes, as explained by Evbuomwan and Chowdhury (2020).

The interaction between pH, electro-conductivity and cellular elements in the female reproductive tract is critical for regulating sperm motility and survival, thereby impacting fertility in animals such as cows (Warr *et al*., 2020). According to Hafez and Hafez (2000), imbalances in acidity or alkalinity in cervical mucus can hinder sperm motility and lead to failed fertilization. Optimal pH levels in cervical mucus, as noted by Fordney-Settlage (1981), are essential for the survival of sperm cells, while deviations beyond this range can impair sperm fertility. Brannigan and Lipshultz (2008) further suggest that a pH range of 7.0-8.5 is ideal for supporting sperm viability and motility.

2.5. Factors Affecting Conception in Dairy Cows

The successful reproduction of dairy cows is vital for dairy farmers aiming to achieve an annual one-calf-per-cow ratio. To ensure successful conception, it is crucial to consider various factors such as herd-level management practices, environmental factors and individual cow characteristics (Hudson, 2011). Elements that can impact conception rates include nutritional intake, breed, age and weight of the cows, their body condition, post-partum heat phase, estrus cycle, timing of insemination, breeding season, as well as anatomical and genetic abnormalities present in both cows and bulls (Ferdousi and Khan, 2013).

2.5.1. Environmental factors

The reproductive efficiency and sexual receptivity of cattle are influenced by environmental factors such as weather, day length, ambient temperature and photoperiod, varying according to the season (Sakaguchi *et al*., 2023). Research indicates that cattle exhibit different estrus behavior patterns depending on the season, showing longer estrus durations in summer compared to winter or spring. Moreover, cows experience more frequent mounting per estrus cycle in winter relative to summer or spring. Therefore, estrous detection may require more regular monitoring in winter and longer duration per check-in during summer (White *et al*., 2002).

High environmental temperatures, known as heat stress, can significantly impact the reproductive behavior of cattle by reducing the vigor and length of estrous expression, ultimately hindering natural mating behavior (Khan *et al*., 2023). This decrease in estrous activity may be linked to lower dry matter intake, which can affect hormone levels in cows (Guimaraes-Yamada *et al* ., 2022). Moreover, temperature fluctuations throughout the day can influence the duration of estrus, with cattle exposed to cooler temperatures potentially exhibiting shorter estrous periods compared to those in warmer conditions (Nohara *et al*., 2022). These findings underscore the detrimental effects of heat stress on cattle reproduction and highlight the importance of mitigating strategies to improve reproductive efficiency and minimize losses in the cattle industry, such as advanced reproductive technologies and environmental management practices (Khan *et al*., 2023).

2.5.2. Management factors

The successful conception in dairy cows depends on various management factors, including husbandry methods, feeding practices, estrus detection techniques, housing conditions, use of skilled inseminators, proper semen handling, correct insemination location and timing of insemination in relation to estrus and ovulation (Lobago *et al*., 2006). These factors play a crucial role in enhancing reproductive outcomes in dairy cattle. Nutritional management is a critical factor in ensuring successful pregnancy, with energy status playing a significant role in reproductive performance. Studies by Smith (1982) and Anzar *et al*. (2003) highlight the importance of total dietary intake in influencing fertility at the oocyte and embryo levels. Boland and Lonergan (2005) and Funstone *et al*. (2009) emphasize that nutrition directly impacts the quality of follicles, oocytes and embryos.

The type of housing can impact the ability to detect estrus in cattle. Research shows that cattle in tie stalls exhibit less estrous behavior compared to those in free stalls (Felton *et al*., 2012). Cattle housed in environments where they can interact freely have more opportunities to display mounting and standing behaviors, making it easier to identify cows in estrus. To accurately detect estrus in cattle housed in tie stalls or stanchion barns, they should be allowed time for these behaviors to be expressed (Lucy, 2001).

According to Lucy (2001), the type of floor surface on which high-producing Holstein cows are housed can impact their expression of estrus. Cows kept on dirt floors exhibit more consistent estrous behavior, including standing and mounting activities, and have longer durations of estrus compared to cows housed on slippery concrete floors. This suggests that the farm environment, specifically the floor surface, can influence estrous behavior and mounting activities in dairy cattle. Elevated environmental temperatures have a detrimental impact on the natural mating behavior of cows, leading to a reduction in both the duration and intensity of estrus. The most common and costly reason for the failure of artificial insemination (AI) programs is inaccurate estrus detection, with cows often being misidentified as being in estrus and inseminated when conception is not possible (Lopez-Gatius, 2011).

Among various factors, the precision of heat detection plays a crucial role in determining the effectiveness of an AI program. Detection of heat in cows is typically performed by skilled herdsmen or inseminators who can recognize animals exhibiting signs of heat. Due to its brief duration between two consecutive estrus cycles, a detailed observation is essential (Arthur, 2001; Anjum *et al*., 2009). Additionally, Arthur (2001) noted that several factors, such as heritability, days postpartum, lactation number, milk production and overall health, can influence the manifestation of estrus.

Table 1. Showing proper timing of insemination

Cows showing estrus	Should be inseminated	To be late for good results
In morning	Same day	Next day
In afternoon	Morning of next day or Early afternoon	After 3 pm next day

Source: (Webb, 2010).

2.5.3. Cow related factors

The success of cow conception is influenced by various factors such as age, body condition score, milk production level, genetics and postparturient issues (Hudson *et al*., 2012). Particularly, the health of the cow's reproductive system, specifically the uterus, plays a crucial role in achieving successful conception. Research indicates that cows with uterine infections after calving experience lower conception rates in subsequent breeding attempts, even with mild infections (Sheldon *et al*., 2009). Ahmadi and Dehghan (2007) also highlighted how health and reproductive problems post-calving can significantly impact conception rates.

The success of conception in dairy cows was influenced by age, with heifers typically having higher pregnancy rates due to lower embryonic mortality compared to older cows. As cows near the end of their reproductive life, there is an increase in embryonic mortality rates. Both early and late embryonic losses rise with age among dairy cattle (Dhamsaniya and Parmar, 2016). The body condition score (BCS) of dairy cows plays a crucial role in their reproductive success. Cows with poor BCS may experience delays in expressing estrous after calving, leading to a lower conception rate (Butler, 2001). Conversely, cows with high BCS, characterized as overweight or obese, may require multiple breedings for conception and show reproductive inefficiencies (Sakaguchi, 2009; Wang *et al*., 2019). Research suggests that the ideal BCS for cows is 3.0 (Loeffler *et al*., 1999).

The negative energy balance (NEB) in cows can be influenced by parity, with primiparous cows producing less milk than multiparous cows due to increased growth requirements. This heightened nutrient demand may exacerbate the NEB severity, impacting the length of postpartum anestrus differently based on the cow's parity. Research by Tanaka *et al*. (2008) suggests that primiparous cows experience longer periods of anestrus compared to pluriparous cows, while Kawashima *et al*. (2006) found that primiparous cows actually have shorter anestrus periods. Additionally, Khan *et al*. (2015) reported higher conception rates in cows at second and third parity than in cows at zero parity.

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted from October 2023 to May 2024 in dairy farms found in Bishoftu Town east Shewa zone of the Oromia region. The area is located at 45 km along southeast of Addis Ababa which is located at 9°N latitude and 40°E longitudes at an altitude of 1850 meters above sea level in central highlands of Ethiopia **(Figure 3)**. The average annual rainfall is 866 mm with a bimodal distribution. The long rainy season extends from June to September (of which 84% of the rain is expected) followed by a dry season from October to February. The short rainy season lasts from March to May. The mean annual minimum and maximum temperatures are 14ºC and 26ºC, respectively. The humidity of the study area is 66% in summer and 56% in winter (CSA, 2017).

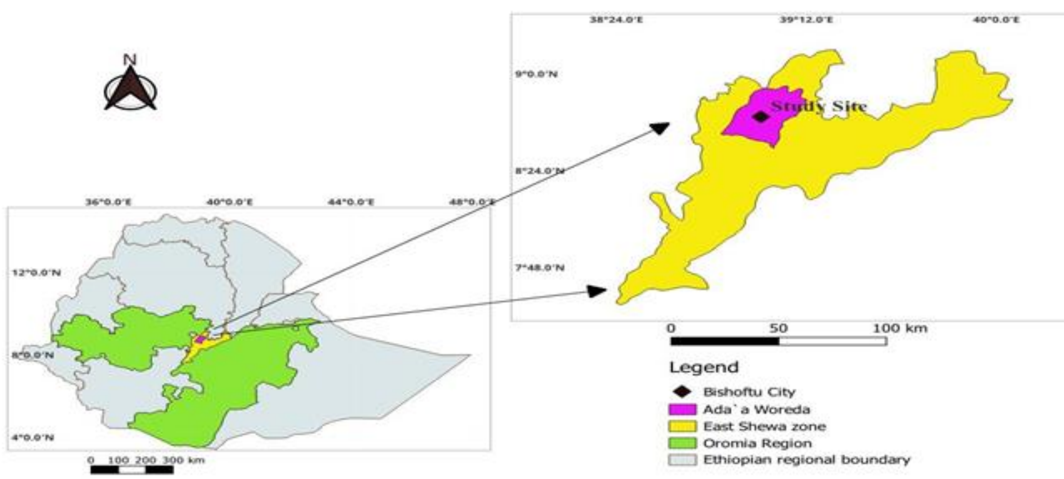

Figure 3. Map of Bishoftu town, east Shewa zone, Ethiopia

3.2. Study Animals

A total of 62 animals were included in this study, of which 38 were normal-cycling cows and 24 were repeat-breeder Holstein-Friesian dairy cows. Normally cycling cows are those cows that conceived normally after a maximum of three inseminations, while repeat breeder cows are those that failed to conceive after more than three successive inseminations. The age of the animals were recorded (ranging from 3 to 9 years), body weight as measured from heart girth ranged from 300 to 450 kg, parities from 1 to 5 and body condition score was considered sub-optimal when < 3 BCS and optimal when ≥ 3 BCS, according to Klopii *et al*. (2011).

3.3. Management of Animals

The animals in all dairy farms visited were typically housed in a similar manner, with concrete floors and tie-stall systems. They were fed a diet consisting of green fodder, grass hay, compounded feed concentrate and mineral mixture according to a standard feeding schedule. Clean water was freely accessible to all animals. Lactating cows were hand-milked twice daily and all animals were identified with long plastic ear tags. Vaccination against common diseases such as Anthrax, Blackleg, FMD and Lumpy skin disease was administered in all farms. Breeding involved artificial insemination, with herd attendants relying on visual observation of heat for AI timing.

3.4. Study Design

A sample survey and follow-up study design was conducted from October 2023 to May 2024 in market-oriented dairy farms found within Bishoftu town. The repeat breeding cows were purposively and conveniently sampled based on the complaint by the farmer while the normally cycling animals were sampled at some haphazard intervals based on the call for AI service. Each time, cows with the repeat breeding complaint were counterchecked for the correctness of the information both from the record and owner interview. All measurements and sampling were taken before insemination.

3.5. Detection of Estrus and Determination of Vaginal Electrical Resistance

The oestrus of cows was detected by the dairy owners based on behavioral and physical manifestations of oestrus, such as restlessness, frequent micturition, bellowing, mounted but not standing, chin resting, mounting other cows, head butting, standing to be mounted, vulvar edema and redness and presence of vaginal mucous discharge. The observation of these signs was confirmed and recorded.

A battery-powered Bovine Draminski oestrus detector (Draminski Electronics in Agriculture, Poland) was used to obtain the vaginal electrical resistivity as described in Agbubga *et al*. (2019). A cow with low resistance readings indicates estrus and hence an eminent ovulation. The measurement of vaginal electrical resistance is a method often used for predicting the optimum time for insemination.

The process of VER recording involved first disinfecting the probe with iodine solution and drying it both before and after each cow's examination. The cow was then secured in a crush, and the perineal area, including the vulva, was cleaned with soap and water before being dried. Subsequently, the vulvar lips were separated and the probe was carefully inserted into the vagina to a depth of 20-25 cm. The probe was gently rotated and moved back and forth a few times before being held in place for 10-15 seconds or until the display readings stabilized. Two VER readings were taken by activating the Ohmmeter button on the probe and the average of these two readings was recorded as the final VER measurement (Purohit and Gupta, 2000).

After each VER determination, the probe was wiped with a clean gauze pad to remove contamination and placed into an iodine solution until the next animal was in place for examination. Before each subsequent measurement, the probe was thoroughly rinsed with water and shaken to remove any excess water. The VER was recorded before the time of AI in all cows and grouped into ranges of 150-180, 181 to 220 and >220 ohms (Ahmed *et al*., 2017).

3.6. Collection of Cervical Fluids

To collect cervical mucus from a cow on the day of estrus before artificial insemination, the following steps were taken: restrain the cow, clean the vulva with disinfectant, insert a catheter through the vagina with rectal guidance to reach the cervix, aspirate mucus samples with a syringe, transfer the fluid to a sterile tube, label the tube with cow information, date and time, and sent to the laboratory for testing on pH, spinnbarkeit, sperm penetration and crystallization pattern.

3.6.1. PH

The pH of cervical mucus was evaluated using a pocket-type pH meter. Initially, place the electrode approximately 2 to 3 cm into the pH standard buffer solution, then transfer it into the cervical fluids. Finally, note the pH reading on the pocket pH meter and classify it into three ranges: $\leq$7, 7.1-7.5 and 7.6-8.0.

3.6.2. Spinnbarkeit test

The spinnbarkeit was measured with a simple apparatus, as described by Tsiligianni *et al*. (2000). A one drops of cervical mucus were placed on a grease-free slide and another grease-free glass slide was placed over it. The slides were slowly moved away from each other to stretch between two slides and the slide was moved until the mucus string broke (**Figure 4**). The distance between the two slides just before the breakage of the mucus string was measured through a scale (cm scale) mounted on the wall, known as the Spinnbarkeit value. This procedure was repeated twice for each sample and the mean value was recorded. Results were expressed in cm. In this study, Spinnbarkeit values were categorized into three groups <7 cm, 7–9 cm and >9cm (Modi *et al*., 2011) .

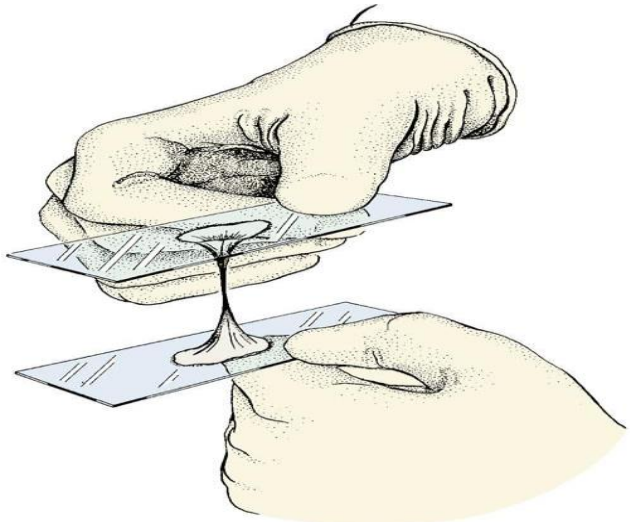

Figure 4. Measurement of spinnbarkeit on cervical mucus using a double slide method

3.6.3. Sperm penetration test

To determine sperm penetration into cervical mucus. Procedurally, first put a small drop of the cervical fluid on a slide (**Figure 5**). Then drop 50µl of frozen-thawed semen placed slightly closer to it. Next, put a cover slide on top until the edges of the two drops contact and wait a few minutes. The final examination under a low power objective (10x) microscope for sperm has passed to the cervical mucus. Results were expressed as normal (many sperms found in the cervical fluid), poor (only a few sperms), or negative (no sperms) (OMS, 2001).

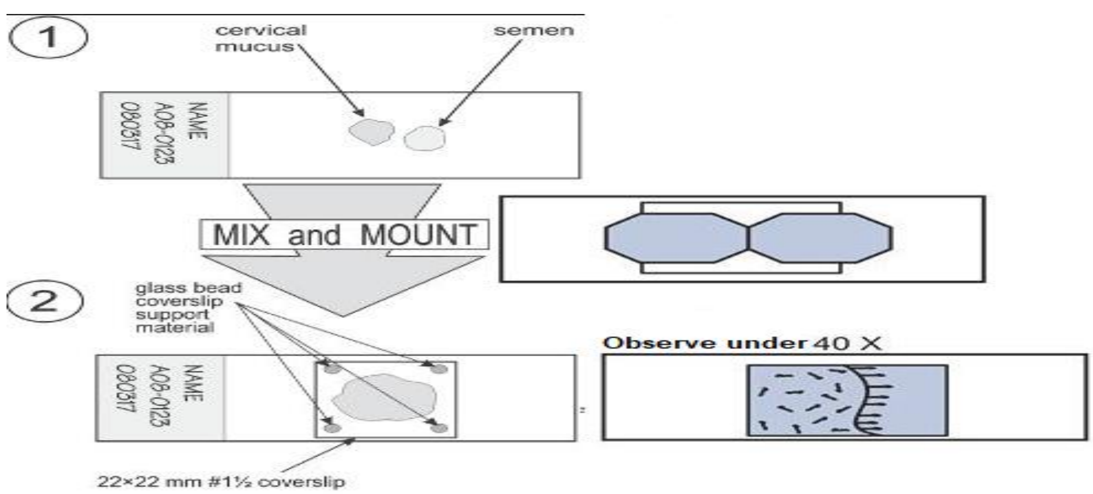

Figure 5. Sperm penetration test

3.6.4. Crystallization pattern

The Crystallization pattern was determined as described by Tsiligianni *et al*. (2000). A film was prepared by smearing a drop of cervical mucus on a clean microscope slide. The film was allowed to dry at room temperature for 30 to 40 min. The air-dried slide was examined under a microscope using a low-power objective (10X) for the crystallization pattern of the mucus. The Crystallization pattern of the observed mucus was classified according to an arbitrary scale proposed by Cortes (2012) on a scale of 0 to 4: 0: No crystal formation, 1: Formation of only atypical crystals, 2: Formation of many atypical and few typical fern-like crystals, 3: Formation of many typical fern-like crystals and few atypical ones, 4: Formation of only typical fern-like crystals.

Figure 6. Typical crystallization pattern of bovine cervical mucus at oestrus observed under light microscopy

3.7. Pregnancy Diagnosis

All inseminated cows were checked for conception after 30 days post-AI using transrectal Ultrasonography (B mode, linear array real-time with 7.5 MHz frequency probe). Cows, which come to heat after 17 to 24 days later to AI, were considered as non-pregnant. Conception rate (CR) was calculated using the following formula:

$$\text{Conception rate (\%)} = \frac{\text{Number of pregnant animals}}{\text{Total number of animals inseminated}} \times 100$$

3.8. Data Management and Analysis

The collected data were grouped according to the age of the animal, BCS, parity, reproductive status, time of insemination, weight, VER, Sperm penetration, Spinnbarkeit values and Crystallization pattern and coded and stored in a Microsoft Office Excel 2010 spreadsheet. Data was summarized using descriptive statistics (means and standard errors) using STATA version 14.0. (StataCorp LP, Texas, USA). In this study, descriptive statistics were calculated for average Spinnbarkeit value, pH and vaginal electrical resistances between normal cycling and repeated breeder cows. Conception rates among groups of age, BCS, parity, weight and cervical fluid characteristics were compared by chi-square test ($\chi 2$). The Logistic regression analysis was used to compare the time of insemination and VER of estrus cows with a relationship to conception rate. P-values were held at < 0.05 to consider the presence of a statistically significant difference.

3.9. Ethical Clearance

All procedures were carried out in accordance with the ethical standards set by the Addis Ababa University College of Veterinary Medicine and Agriculture following approval from the ethics committee (Reference No: VM/ERC/03/16/024, 16/03/2024; see Annex 6).

4. RESULT

4.1. Physico-Biochemical Properties of Utero-Vaginal Secretion in Dairy Cows during the Estrus Period in Relation to Fertility

pH of cervical fluid in this data did not show any association with conception rate although slightly alkaline condition seemed to have favoured relatively higher conception rate (Table 2). Spinnbarkeit values were known to have significantly affected ($P<0.05$) conception rates. Cows with a spinnbarkeit value with the highest conception rate (87.5%) recorded for cows between 7-9 cm. Details of findings on conception rates were indicated in the table below(Table 2).

Table 2. The effects of characteristics of cervical fluid on conception rate (n=62)

Cervical fluid characteristics	Category	Conception rate (%)	P-value
pH	≤7	50.0 %	
	7.1-7.5	60.0%	0.719
	7.6-8	47.1%	
Spinnbarkeit value(cm)	<7	43.9%	
	7-9	87.5%	**0.009**
	>9	40.0%	
Sperm penetration	Poor	30.8%	**0.00**
	Normal	72.2%	
Crystallization	Typical	76.47%	**0.015**
	Atypical	23.53%	

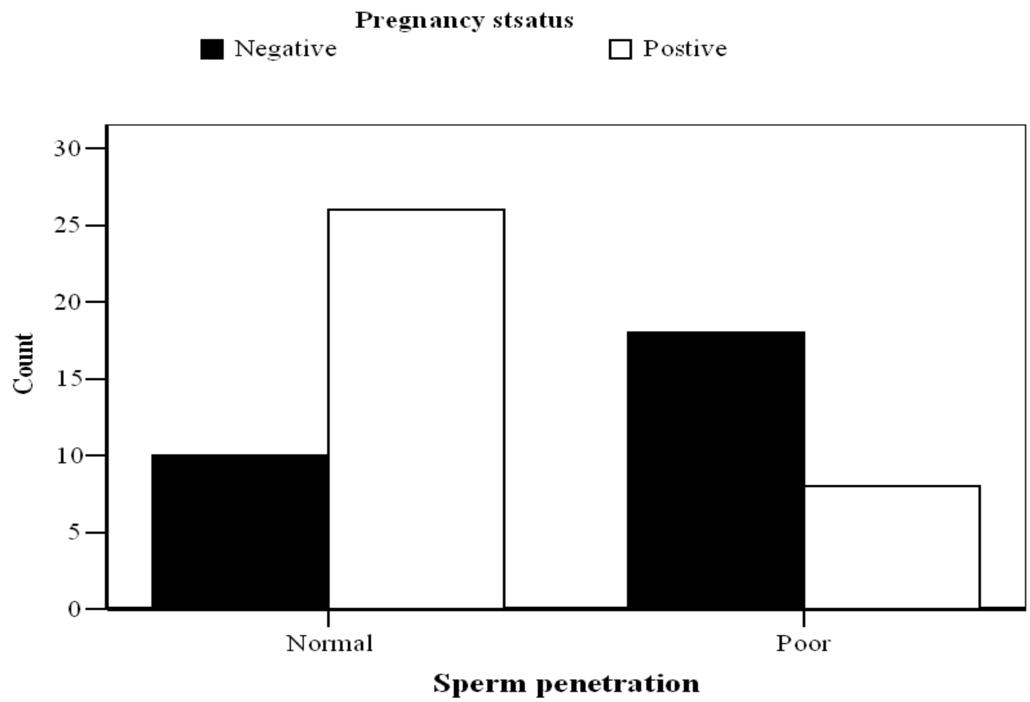

Figure 7. Sperm penetration test values between pregnant and nonpregnant cows

Figure 7 shows cows with normal sperm penetration exhibited a significantly higher positive pregnancy status, with 26 out of the 36 counts resulting in pregnancy. In contrast, cows with poor sperm penetration had lower positive pregnancy outcomes, with only around 8 out of the 26 counts resulting in pregnancy.

The mean pH and spinnbarkeit value of cervical fluid of pregnant cows were 7.33±0.26 and 6.53±2.14 cm, respectively. More details are presented in (Table 3).

Table 3. The mean scores of cervical fluid characteristics in pregnant and non- pregnant cows.

Variables	pregnant (mean±SE)	Non-pregnant (mean±SE)
pH	7.33±0.26	7.39±0.35
Spinnbarkeit value (cm)	6.53±2.14	4.9± 3.41
Vaginal electrical resistance (Ω)	202.15± 31.15	241.79±73.56

4.2. The Influence of Cow's Factors in the Success of Conception Rate

The overall conception rate was 54.84 % (34/62) and there was a significant difference between the different body weight categories with the highest conception rate recorded for cows weighing between 351 and 400 kg (Table 4). However, age, BCS, and parity did not significantly correlate with the conception rate**.**

Table 4. The influence of cows' age, BCS, parity, and weight on conception rate.

Variables	**Categories**	**N**	**Conception rate (%)**	χ^2	**P-value**
Age (year)	3-4	28	46.43	1.85	0.40
	4.1-6	24	58.33		
	>6	10	70.00		
BCS	<3	18	44.44	1.11	0.29
	≥3	44	59.09		
Parity	1	26	42.31	4.51	0.11
	2-3	29	58.62		
	≥4	7	85.71		
Weight(kg)	300-350	16	31.25	14.40	**0.001**
	351-400	36	75.00		
	401-450	10	20.00		

Key: BCS: Body condition score

4.3. The Association between Conception and Factors around the Time of Estrus

The conception rates of cows inseminated 0–6 hours, 7–12 hours, and >12 hours after standing heat were 30.4%, 71.43%, and 50.0%, respectively (Table 5). The probability of cows becoming pregnant was 15.95 times higher when the cows were inseminated 7-12 hours (P-value = 0.019) after the standing heat compared to cows inseminated earlier (0-6 Hours). Comparatively, cows inseminated at a later stage (>12 hours) still had a higher chance (3.59 times more) of becoming pregnant (P-value = 0 .480) than those inseminated at an earlier stage, even though there was no statistically significant difference between the two groups in terms of conception rate (Table 5).

A highly significant difference ($p < 0.05$) was observed in the conception rate between cows with different VER measurements. The conception rate was 66.67%, 84.21%, and 24.00% in cows with VER of 150-180 Ω, 181-220 Ω and >220 Ω, respectively. The chances of conception were 58.48 and 0.105 times higher for cows with VER values of 181-220 Ω (P-value=0.029) and >220 Ω (P-value= 0.036) , respectively as compared to cows with 150-180 Ω VER (Table 5).

Table 5. The effect of VER and time of insemination on conception rate

Parameters	Category	N	Conception rate(%)	OR	P-value
Time of insemination (hr)	0-6	23	30.43	Ref	
	7-12	35	71.43	15.950	**0.019**
	>12	4	50.00	3.559	0.480
VER(Ω)	150-180	18	66.67	Ref	
	181-220	19	84.21	58.480	**0.029**
	>220	25	24.00	0 .105	**0.036**

***Key**: VER-Vaginal electrical resistance*

4. 4. Differences in Physico-Biochemical Properties of Cervical Mucus between Normal Cycling and Repeat Breeders Dairy Cows

The mean VER of the cows showing normal and repeated breeding was 219.03 ±66.17Ω and 221.67±41.77Ω, respectively. The mean pH and SBK for normal cycling were 7.38±0.32 and 5.76±2.2cm and 7.32±0.27 and 5.85±3.77cm for repeat cows (Table 6).

Table 6. The average score of vaginal electrical resistance(VER), PH and spinnbariket valve (cm) in normal cycling and repeated breeder dairy cows

		Mean (±SE)		
Reproductive status	**N**	**VER (ohm)**	**PH**	**SBK(cm)**
Normal-cycling	38	219.03 ±66.17	7.38±0.32	5.76±2.20
Repeat breeder	24	221.67±41.77	7.32±0.27	5.85±3.77
Total	62	220.05±57.58	7.36± 0.30	5.80±2.88

Key: *SE = Standard error of the mean, VER- Vaginal electrical resistance, SBK- Spinnbarkeit*

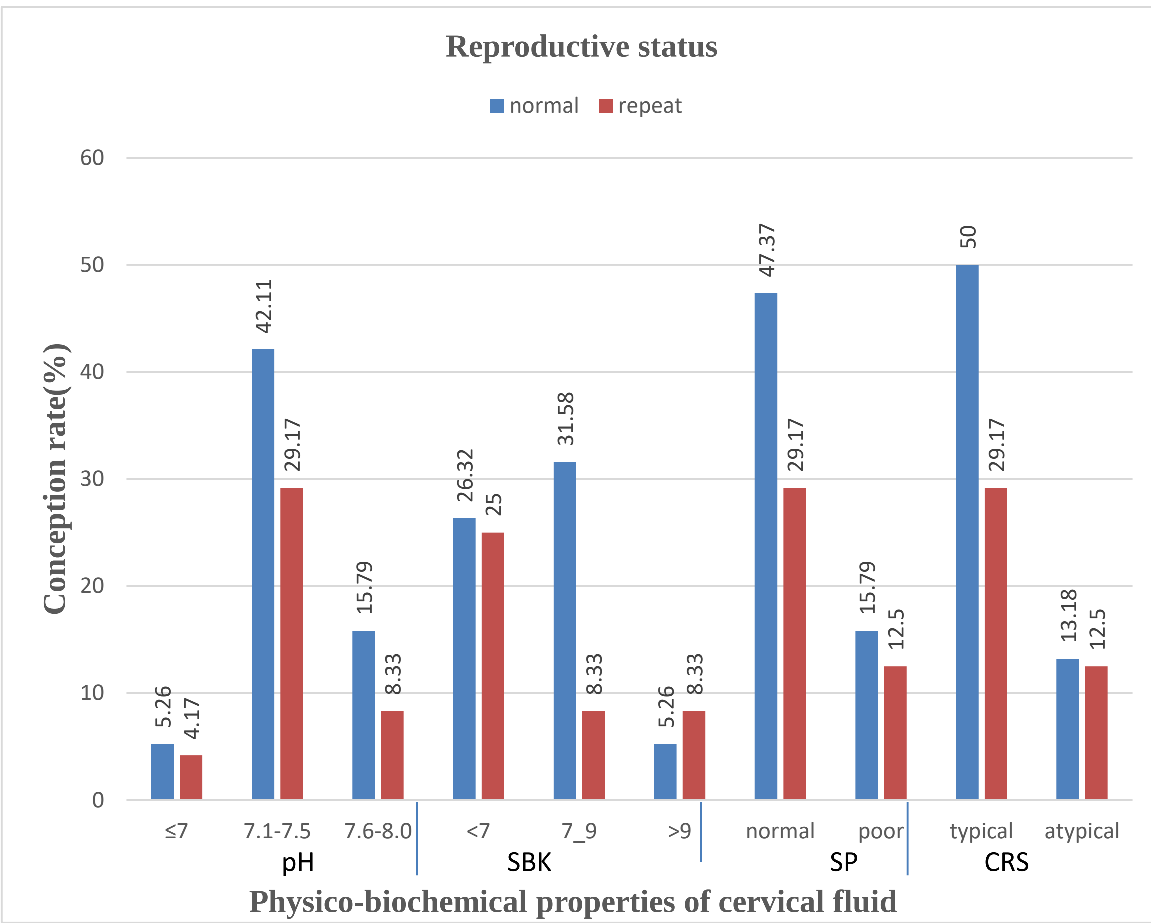

Figure 8. Physico-biochemical properties of cervical fluid between normal cycling and repeat breeders Holstein Friesian dairy cows.

Key: *SBK-Spinnbarkeit ; SP- Sperm penetration; CRS-Crystalization*

Figure 8 shows that the cows with a typical cervical fluid crystallization pattern (50%) had a greater conception rate among the normal cycling cows than the repeat breeders. Repeat breeder cows with acidic cervical fluid (pH ≤ 7) had the lowest conception rate compared to the other physico-biochemical properties of cervical fluid examined in the repeat breeder group.

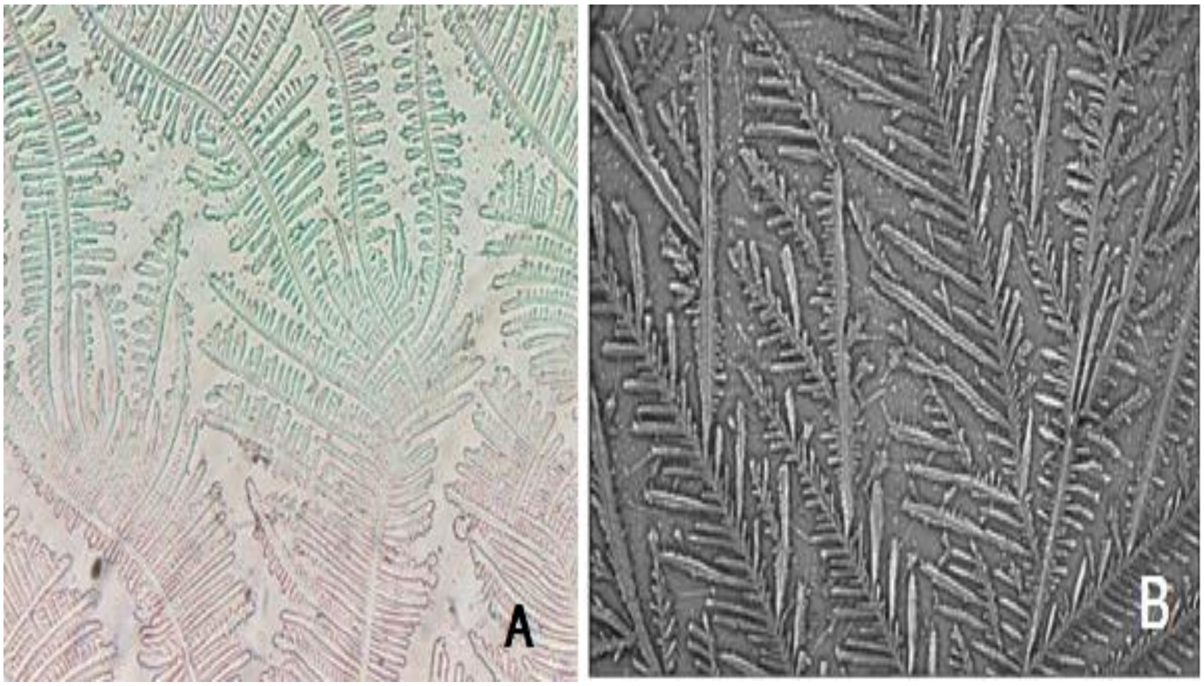

Figure 9. Atypical (A) and Typical (B) crystalization pattern of cervical mucus in Holstein cows during estrus

5. DISCUSSION

The present study reports the evaluation of the physico-biochemical characteristics of cervical fluid (cervico-vaginal mucus) in dairy cows during the estrous phase in association with conception. The characteristics of cervical fluid are important indicators for fertility prediction in cattle (Jeong *et al*., 2010). Cervical fluid, a substance primarily generated by the cervix, plays a significant role in cow's pregnancy outcomes. Changes in cervical mucus composition during hormonal changes in the ovaries facilitate sperm movement. Various parameters like the pH, spinnbarkeit, sperm penetration ability, crystallization patterns and electrical conductivity have been used to determine the most suitable time for cattle insemination. This information has been reported in studies by Jeong *et al*. (2010), Ondho *et al*. (2019), Siregar *et al*. (2019) and Abd-El-Hafeez *et al*. (2020).

The overall conception rate found in the current study is in agreement with previous reports for HF dairy breeds by Shamsuddin *et al*. (2001) and Mollah (2011), who also reported rates of 54.9% and 55.1%, respectively. These rates were however, lower than the rates reported by Shiferaw (2002) at 84.7% and Arthur (2001) between 63-71%. The variation in reports can be due to several factors like identifying estrus, the health of the animal, the proficiency of the inseminator, timing of insemination after estrus and quality of semen. In tropical regions, cattle experience lower fertility rates compared to those in temperate areas due to factors like poor nutrition, higher disease rates and the complex interplay between genetics and the environment (Mukasa-Mugerwa, 1989).

The present study found that the conception rate of cows with suboptimal body condition (<3) was 44.44%, while cows with optimal body condition (≥ 3) had a conception rate of 59.09%. Body condition did not significantly impact the conception rate, which agrees with previous research by Perry *et al*. (2003) and Navanukraw *et al*. (2004). In contrast, earlier studies by Ciptadi *et al*. (2012) and Tazangi and Mirzaei (2015) suggested that body condition influenced conception rates, with higher rates in animals with a higher body condition score. The lack of association in the present study's results could be attributed to limited variation in the body condition of the animals included.

Regarding the timing of insemination, it is an established fact that the length of time between standing oestrus and insemination affects the conception rate in dairy cows (Gonzalez, 1981; Jemal and Lemma, 2015; Hamid *et al*., 2021). The current findings that agrees with those of Jemal and Lemma (2015), confirms a higher conception rate when cows were inseminate within 7–12 hours of the standing heat. The variation is often a result of mismatch between the times of ovulation and insemination. This further underscores the necessity for breeding early enough for capacitation and late enough so fertile life span of the sperm and oocyte overlap. Many authors also emphasize the importance of timely insemination for improving conception rates in cows (Arthur, 2001; Ball and Petters, 2004; Reolofs *et al*., 2010).

A highly significant difference was observed in the conception rate at varying VER in the present study. The fact that the chances of conception were many folds higher for cows with 181-220 Ω of VER value in comparison to those cows with either very low or very high VER means such measurement could be a more accurate predictor of pregnancy success even when overt estrus signs are absent. The current finding also aligns with previous research by Ahmed *et al*. (2017), which also showed higher pregnancy rates in cows with closely similar patterns of VER.

Some research has shown that inseminating cows with a low VER of 26 to 30 Ω using the Ovotec, which is a different device than the one used in this study has led to higher conception rates ranging from 84.00% to 92.50% (Purohit and Gupta, 2000; Meena *et al*., 2003). Tadesse *et al*. (2011) and Meena *et al*. (2003) also reported lower VER values during estrus in Ethiopian dairy cattle and Indian Rathi cows using the same Ovatec device. In any case is important to emphasize that VER levels can be used to determine the best time for AI in cows to improve conception rates. Contrary to this, studies by Zuluaga *et al*. (2017) indicates that VER was not a reliable method for predicting the ideal time for insemination following estrus induction and synchronization in cows, particularily in large farm settings.

Values of the VER in pregnant and non-pregnant cows found in this study is also in agreement previous report by Ahmed *et al*. (2017). Variations in VER values during estrus may stem from factors such as differences in intravaginal probe design, estrus cycle stages, probe insertion depth and positioning, pressure applied on the vaginal mucus, genital tract conditions, electrode probe insertion issues and hand pressure disparities during resistance measurements (Gupta and Purohit, 2001).

Examining the relationship between estrual cervical mucus characteristics and fertility in cows, it was noted that cows with a cervical fluid pH level between 7.1 and 7.5 exhibited a higher conception rate compared to cows with cervical mucus that has more acidic or alkaline nature. Similar findings have been reported by Abd-ElHafeez *et al*. (2020), who observed a higher conception rate of 42.86% in Egyptian Baladi cows with a vaginal mucus pH within the range of 7.0 to 7.5. However, this contrasts with the earlier study by Damarany (2020) where a higher conception rate of 67.5% was found in crossbred cows with a vaginal mucus pH of 8.14 as opposed to those with a pH of 7.46. Additionally, Verma *et al*. (2014) reported a higher conception rate of 42.2% in Murrah buffaloes with a vaginal mucus pH level at estrus ranging from 7.5 to 8 compared to cows with a pH range of 7 to 7.5 at 25%.

The pH value of 7.33±0.26 in non-conceived cows closely resembled the findings of Siddiquee (2006) at 7.40±0.09. In contrast, Kumar *et al*. (2019) observed that the vaginal mucus pH was 8.42±0.09 for conceived cows and 8.72±0.22 for non-conceived crossbred cows. Bennur *et al*. (2004) and Rathod (2016) recorded pH levels of 8.13±0.07 and 8.45±0.11 in cervical-vaginal mucus of conceived cows, respectively. Hafez and Hafez (2000) suggested that the increased acidity or alkalinity in cervical mucus could weaken sperm motility and lead to fertilization failure.

Variations in pH levels were likely attributed to the specific measurement techniques employed and variations in estrogen levels among individual cows. Suharto (2003) suggested that environmental factors present during measurement could also impact cervical mucus pH. For instance, when cervical mucus pH was measured externally (in vitro) in high-humidity conditions, it tended to show increased alkalinity. Additionally, exposure of cervical mucus to atmospheric air for a brief period (5-10 minutes) can also influence its pH levels (Correa *et al*., 2001). Studies have shown that the pH of the vagina during the course of fertilization may influence the migration of X- and Y-bearing spermatozoa thus leading to skewness in the sex of the offspring (Raval *et al*., 2019).

The findings of average pH levels of cervical fluid in normal cyclic cows and repeat breeder cows align with previous studies by Pandey *et al*. (1983) which also reported a pH of 7.38 for normal cows. In contrast, Gohel *et al*. (2012) found a slightly higher pH of 7.17±0.04, while Modi (2007) reported a much higher pH of 8.39. Similarly, the pH of cervical fluid in repeat breeder cows matched closely with the findings of Gohel *et al*. (2012) at 7.22±0.04 and was notably higher than the pH of 6.19 reported by Modi (2007).

The normal breeder showed higher PH as compared to repeated breeder cows. Alkaline PH of cervical fluids was more favorable for sperm progressive motility (Rangnekar *et al*., 2002). However, a non-significant difference in the PH secretion was obtained from normal cycling and repeated breeder cows. Various studies, in general, show impact of pH on sperm motility, viability and capacitation which can be the cause for differences in conception and skewness of sex, but alteration of pH can occur at both the male and female level, ie in semen samples as well as in vagina of female.

Cows with a spinnbarkeit value have the highest conception rate recorded for cows between 7 and 9 cm compared with cows with cervical mucus measure of spinnbarkeit value of <7cm or >9cm. This is consistent with a study by Modi *et al*. (2011) showing that Kankrej cattle primarily had values of 7-9 cm during estrus, unlike findings from Parikh *et al*. (2018) in Gir cows where values were mostly >16 cm. The results of this study indicated a possible association between conception rate and spinnbarkeit of cervical mucus at the time of insemination.

The average spinnbarkeit measurement in the cervical fluid of pregnant cows was found to be lower than the measurements reported by Hanumant *et al*. (2019), 13.09±0.40 cm in cows and Sharma *et al*. (2008), 10.30±0.93 cm in buffaloes, but slightly closer to the results reported by Bennur *et al*. (2004), 7.38±0.56 cm in cows. Pregnant animals exhibited a notably higher average spinnbarkeit score of estrus mucus (6.53±2.14) compared to non-pregnant animals (4.9±3.41). This is consistent with earlier work conducted by Yildiz (2021). In contrast, earlier studies by Gohel *et al*. (2012) spinnbarkeit value was higher in cows that failed to conceive (13.58±0.08) as compared to conceived (13.03±0.50) cows that were induced for estrus.

Value spinnbarkeit in cervical mucus changes as a result of blood estrogen levels which is also responsible for behavioral manifestation of estrus. The average spinnbarkeit measurement was higher in repeat-breeder cows than those with normal breeding cyclic cows. This finding is in line with Siddiquee (1980), who observed a significantly higher mean spinnbarkeit value in repeat breeder cows (21.10±0.66 cm) compared to normal breeder cows (16.70±4.31 cm). In contrast, Modi (2007) reported spinnbarkeit values of 15.30 cm for normal cyclic cows and 8.0 cm for repeat breeder cows.

Spinnbarkeit value and arborisation/crystallization pattern are the most important properties in relation to fertility in cow. These properties vary with endocrinological status of reproductive cycle and directly associated with the estrogen: progesterone ratio and fertility status of dairy animals. The rheological properties of cervical mucus becomes more plentiful, watery and less viscous, during follicular phase, which facilitates the transport of spermatozoa in the female reproductive tract. However, it becomes opaque, thick, viscous and scanty during luteal phase not favorable to sperm migration (Verma *et al*., 2014).

The conception rate in cows was significantly influenced by the sperm penetration of cervical fluid, with cows having normal sperm penetration exhibiting a higher conception rate compared to those with poor sperm penetration. This finding was similar to several investigators (Keel and Schalue, 2000; Kasimanickam *et al*., 2006) reported that there was a statistically significant between the result of the sperm penetration and the bovine cervical mucus. The lack of penetration by spermatozoa may be due to inadequate quality of the cervical mucus, the presence of sperm antibodies, or faulty kinetics of the sperm cells (Lonergan and Fair, 2016).

The conception rate in cows was significantly influenced by the crystallization pattern of cervical fluid, with cows having typical crystallization pattern exhibiting a higher conception rate compared to those with an atypical crystallization pattern. Similar findings have been reported by Kumaresan *et al*. (2009), Layek *et al*. (2013) and Ningwal *et al*. (2018). However, this contrasts with the earlier study by Alena *et al*. (2008) where a higher conception rate was found in cows with an atypical crystallization pattern.

A typical crystallization was more common in pregnant cows compared to those having atypical crystallization. This finding aligns with a prior study by Bennur *et al*. (2004) which also showed a higher occurrence of typical crystallization in pregnant cows at 87.50% versus 12.50% for atypical and 80.00% versus 20.00% in non- pregnant cows. The occurrence of crystallization is common to all types of mucus. But degree of crystallization/ arborisation pattern in cervical mucus is under the control of two ovarian hormones, estrogen and progesterone. The phenomenon of crystallization in cervical mucus progresses under the influence of estrogen whereas progesterone diminishes the formation of arborisation pattern. However, Selvaraj *et al*. (2002) reported different percentages, with 57.80% showing an atypical pattern and 42.10% showing a typical pattern. Cervical mucus can crystallize due to the presence of mucoproteins, organic compounds and electrolytes like NaCl, KCl and CaCl, with sodium chloride being the predominant salt in cervical mucus according to Cortes *et al*. (2014).

These results are consistent with previous research demonstrating the value of evaluating VER, sperm penetration and cervical fluid characteristics for improving reproductive management in dairy cattle. Studies by Smith *et al*. (2015) and Jones *et al*. (2020) reported similar associations between these parameters and enhanced conception rates in Holstein and Jersey cows, respectively. Additionally, the lack of significant effects from age, body condition and parity aligns with findings from earlier investigations by Brown (2018) and Daisy (2021).

6. CONCLUSION AND RECOMMENDATIONS

The study evaluated the biochemical characteristics of cervical mucus during estrus on conception rates in dairy cows. The results indicate that certain cervical and vaginal parameters can be useful predictors of optimal insemination timing and fertility outcomes in Holstein cows. Specifically, vaginal electrical resistance (VER), sperm penetration of the cervical mucus, spinnbarkeit values and the crystallization pattern of the cervical fluid were found to be significantly associated with conception rates. However, cervical fluid pH did not have a significant effect. Additionally, factors like body weight, timing of insemination and vaginal electrical resistances also impacted conception rates, with the highest rates observed for cows weighing 351-400 kg, inseminated 7-12 hours after onset of standing estrus and having a VER of 181-220 Ω. Although there were some relative tendencies, the effects of factors such as age, body condition score, and parity on conception rate were not statistically significant.

Based on the findings of this study, it is recommended that :

- Dairy farmers and veterinarians consider routinely assessing the physico-biochemical characteristics of the cervical mucus before AI to get an optimal pregnancy.
- Monitoring these cervical and vaginal parameters can provide valuable insights into the fertility status of the cow and help guide more informed breeding decisions.
- Further research is also warranted to validate these findings and explore the underlying physiological mechanisms linking these parameters to fertility.
- Implement proper management practices, including nutrition, heat detection and insemination protocols, to optimize reproductive efficiency and minimize repeat breeding in dairy cows.

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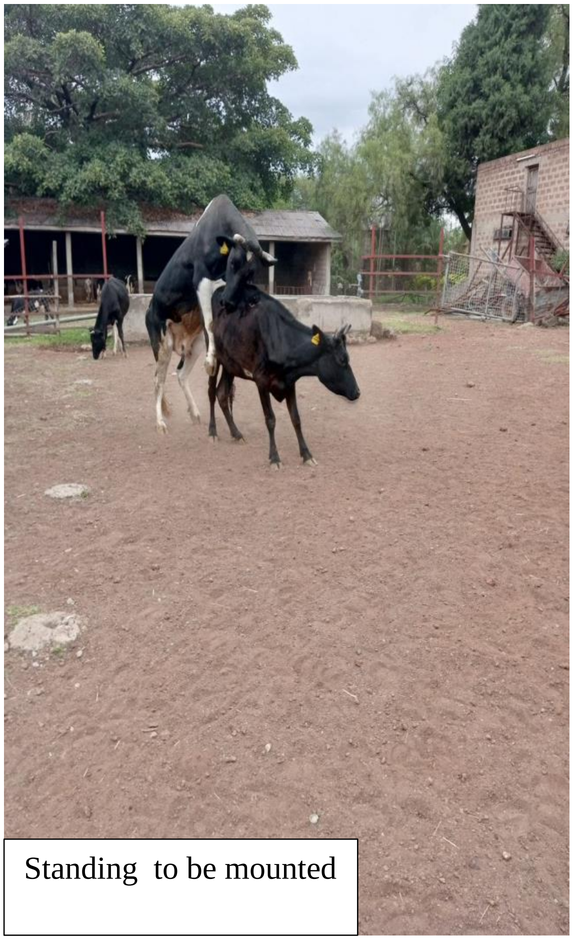
8. ANNEXES

Annex 1. Photography of behavioral estrus signs observed



Standing to be mounted

Sniffing

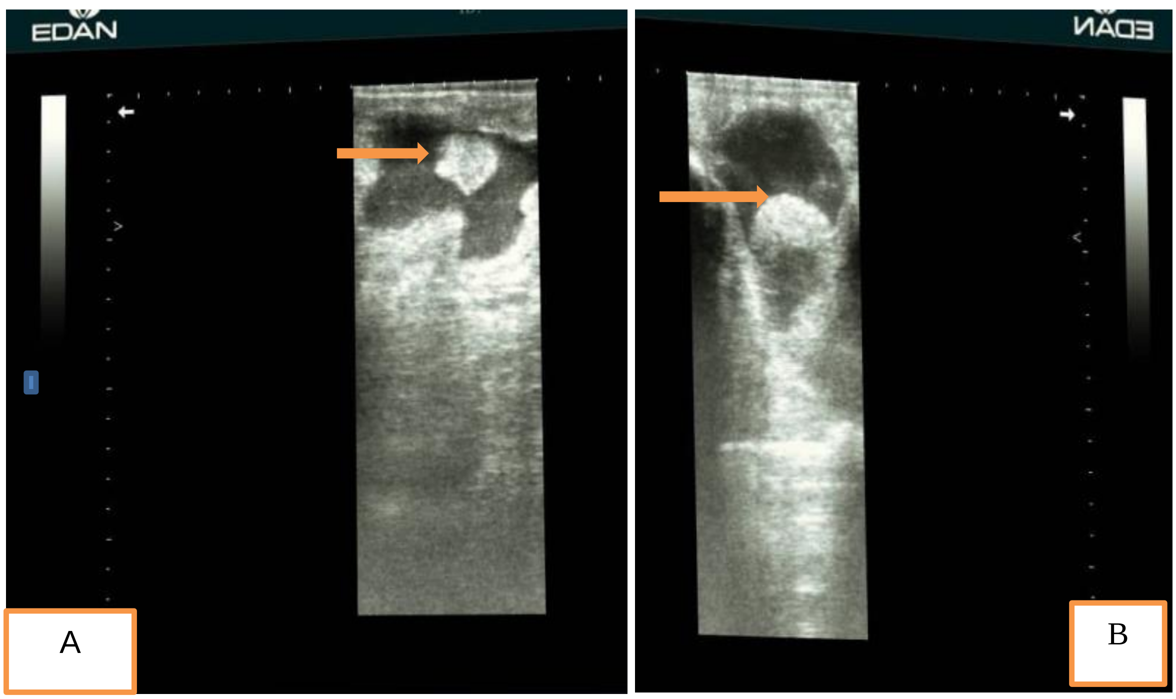
Annex 2. Photography during the sample collection

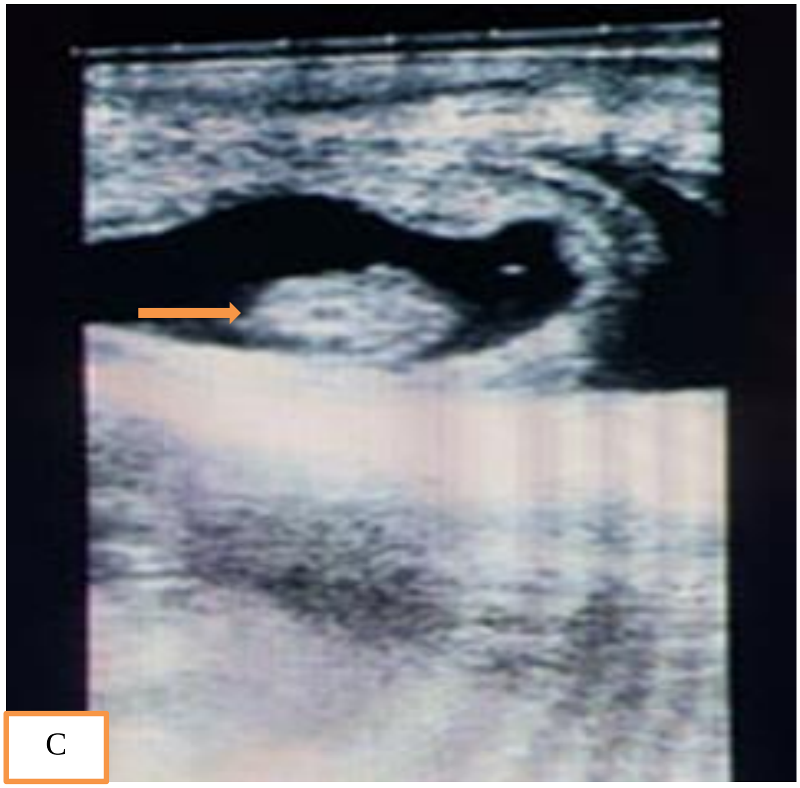
Annex 3. Photography of laboratory work

A. Image of measuring PH of cervical fluid by PH pocket meter
B. Measuring spinnbarkeit value by ruler
C. Observing crystallization pattern under microscope

Annex 4. Photo captured during pregnancy diagnosis by ultrasound in the cows

Annex 5.Transrectal ultrasonographic images of early bovine embryos scanned at days 33, 35 and 40 post inseminations (Red arrow)





A= Ultrasound Image scanned at 33th day of pregnancy, B= Ultrasonic Image scanned at 35th day of pregnancy C= Ultrasound Image scanned at 40th day of pregnancy

Annex 6. Ethical clearance approval certificate

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/03/19/16/2024

Name of Applicant: **Kutelo Kussia (DVM, MSc student)**

Address: Department of Clinical Studies, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Evaluation of biochemical determinants of successful conception in dairy cows in and around Bishoftu, Ethiopia*

Date of application:	**December, 2023**
Nature of the project:	**Farm investigation**
Target animal species:	dairy **cattle**
Number of animals involved:	**62**
Study area:	**Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/03/16/024, 16/03/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Kutelo Kussia.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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Please quote Our Ref. No. When replying

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