Thesis Ref. No._________

EPIDEMIOLOGY OF SUBCLINICAL HYPOCALCAEMIA AND ITS METABOLITE PROFILE IN DAIRY COW DURING TRANSITION PERIOD IN AND AROUND BISHOFTU TOWN, EAST SHEWA ZONE, ETHIOPIA

MVSc THESIS

BY

MOGOS MEKONNIN JIMA (DVM)

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF VETERINARY CLINICAL STUDIES, MVSc PROGRAM IN VETERINARY EPIDEMIOLOGY

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BISHOFTU, ETHIOPIA

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MVSc Thesis

By

Mogos Mekonnin Jima (DVM)

A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Epidemiology

June 16, 2022

Bishoftu, Ethiopia

Approval Sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As Members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by Mogos Mekonnin Jima entitled: **EPIDEMIOLOGY OF SUBCLINICAL HYPOCALCAEMIA AND ITS METABOLITE PROFILE IN DAIRY COW DURING TRANSITION PERIOD IN AND AROUND BISHOFTU TOWN, EAST SHEWA ZONE, ETHIOPIA** and recommended that it be accepted as fulfilling the thesis requirements for the Degree of Master of Veterinary Science in Veterinary Epidemiology.

Dr. Fufa Abuna	________	__________
Chairman	Signature	Date
Dr. Boja Endebu	__________	________
External Examiner	Signature	Date
Dr. Tefera Yilma	__________	__________
Internal Examiner	Signature	Date

June 16, 2022

Bishoftu, Ethiopia

Approval Sheet

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As a Colleague Advisor/s of the final MVSc open defense, we certify that we have read and approved the thesis prepared by Mogos Mekonnin Jima entitled**: EPIDEMIOLOGY OF SUBCLINICAL HYPOCALCAEMIA AND ITS METABOLITE PROFILE IN DAIRY COW DURING TRANSITION PERIOD IN AND AROUND BISHOFTU TOWN, EAST SHEWA ZONE, ETHIOPIA** and recommended that it be accepted as fulfilling the thesis requirements for the Degree of Master of Veterinary Science in Veterinary Epidemiology.

Yitbarek Getachew (DVM, PhD, Asso. Prof.)	__________	___________
Major Advisor	Signature	Date
Haileleul Nigussie (DVM, MVSc, PhD, Asso. Prof.)	_____________	_________
Co-Advisor	Signature	Date
Haileleul Nigussie (DVM, MVSc, PhD, Asso. Prof.)	_____________	_________
Department head	Signature	Date

June 16, 2022

Bishoftu, Ethiopia

DEDICATION

I dedicated this thesis manuscript to my beloved Mom Baay'ise Urgesa, who passed away by leaving her eternal reminiscence to me when I was start work after graduation from WU and my family especially My Son Obsinan Mogos who left from car accident and giving me pleasure to do this manuscript.

STATEMENT OF AUTHOR

First, I declare that this thesis is my original work and that all sources of material used for this manuscript have been acknowledged. This thesis has been submitted in partial fulfillment of the requirements for a postgraduate (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 hardly declare that this thesis is not submitted to any other institution elsewhere for the award of any academic degree, diploma or certificate.

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Name: Mogos Mekonnin Jima (DVM)

Signature: ______________

Place: Addis Ababa University, College of Veterinary Medicine and Agriculture, Bishoftu

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LIST OF ABBREVIATIONS

1, 25-$(OH)_2$ D_3	Dihydroxy vitamin D_3
APHRLC	Adama Public Health Research and Referral Laboratory Center
BAPTA	1, 2-Bis (2-Amino phenoxy) ethane-N, N, N′, N′-tetra acetic acid
BCS	Body Condition Score
Ca^{2+}	Calcium ion
Ca	Calcium
CAPSO	3-[Cyclohexylamino]-2-hydroxy-1-propanesulfonic acid
CSA	Central Statistical Agency
CI	Confidence Interval
DCAD	Dietary Cation Anion Differences
DA	Displaced Abomasum
EDD	Estimated Date of Delivery
EDTA	Ethylene di-amine tetra acetic acid
EB	Energy Balance
ETB	Ethiopian birr
FOA	Funding Opportunity Announcement
QGIS	Quantum Geographic Information System
ILRI	International Livestock Research Institute
IV	Intravenous
MF	Milk Fever
mmol/L	millimoles per litre
NEB	Negative Energy Balance
NM	5-Nitro-5’-Methyl
PTH	Parathyroid Hormone
RFM	Retained Fetal Membrane
RP	Retained Placenta
SCH	Subclinical Hypocalcaemia

ABSTRACT

In order to sustain the healthy, production and productivity of dairy farm, it's important to manage and control the metabolic disease of dairy cow in dairy sector. Hypocalcaemia is one of the most important metabolic diseases of dairy cows that cause significant economic loss in the dairy industry. However, more studies have not been conducted in Ethiopia on subclinical hypocalcaemia during transition period. Therefore, a cross-sectional study was conducted from November, 2021 to June, 2022 in and around the Bishoftu town of East Shewa Zone to know the Epidemiology of subclinical hypocalcaemia and its metabolite profile in dairy cows during the transition period. During the study period, 43 dairy farms were purposively selected based on the willingness of dairy owners. 176 blood samples of dairy cows were sampled from the target population during the study. A semi-structured questionnaire survey was also conducted in parallel to sample collection on dairy farm owners or producers during the study period. The Cobas 6000 electrolyte analyzer at Adama Public Health Research and Referral Laboratory Center was used to measure serum calcium concentrations of sampled dairy cows during transition period. Cows with serum calcium levels < 2.15mmol/L were considered to have subclinical hypocalcaemia, whereas those with serum calcium levels > 2.15mmol/L were considered to have normocalcaemia. The number of dairy cows that were below the threshold point was (n=49) in the study period. The current study revealed that, the prevalence of subclinical hypocalcaemia was 27.8% (CI, 95% = 22% - 35%). The average serum calcium level of sampled dairy cow was 2.174mmol/L (median = 2.21mmol/L), the minimum and maximum value 1.21mmol/L, 2.49mmol/L, respectively. The test of significance association implies that serum calcium concentration was related to breed (local, coef =0.098740, p =0.001), age (coef = -0.042506, p = 0.000137), milk yield (coef = -0.054521, p = 0.042397), feed type (mixed, coef = 0.116441, p=0.002, roughage, coef = 0.091068, p = 0.0049), parity (coef = -0.070485, p = 0.01632) and cow status (pregnant, coef = 0.055772, p = 0.0469). Despite the fact that the farm owner unaware of its presence, the current investigation revealed that subclinical hypocalcaemia is the most prevalent metabolic condition affecting dairy cows in the study area. Therefore, emphasis on host-related and management factors during the transition period is warranted.

Keywords: *Bishoftu, Dairy cow, Epidemiology, Serum calcium, Subclinical hypocalcaemia, Transition period*

1. INTRODUCTION

Development of the dairy industry is a crucial strategic area for addressing food security and improving livelihoods in developing nations, including Ethiopia (Tolosa *et al.*, 2021). According to CSA (2020), Ethiopia has a total of 65.4 million cattle, of which local breeds account for 54.4% and hybrid and exotic breeds makes about 1.19% and 0.21% of the cattle population, respectively. Approximately half (n = 36.5 million) of the cattle population are females (cows), which confers potential for dairy industry development. Dairy cows play a great role in reducing poverty by alleviating the economic crisis of the world and generating regular income for small-holder dairy farms (FAO, 2010).

Smallholder dairy production is an emerging sector in Ethiopia which is providing employment opportunities for youths and women. Dairy farming is a means of income generation and protein source at household level and there of contributing for societal empowerment and food security (Ahmed *et al.*, 2004). There is an increasing trade of the smallholder dairy farms particularly in regional and zonal cities, because of high demand of milk and milk product in relation to the increasing human population (Tadesse, 2018).

Smallholder dairy farms are characterized by their rapid expansion, strong market oriented in rural areas, and the many opportunities for increasing the current level of production. However, the existing farming system which holds maximum of 10 or 15 cows per individual is unsatisfactory to fulfill the increasing demand for dairy products (Devendra, 2001). The major problems affecting milk production potential of dairy cattle in Ethiopia are shortage of grazing land, Infectious and parasitic diseases, shortage of land for cultivation of improved forage, inadequate veterinary service, low milk production potential of local zebu cattle, inadequate Artificial Insemination service and labor shortage (Alemneh, 2019).

In effort to increase productivity of the dairy farms, the government is undertaking crossbreeding programs to boost the genetic potential of indigenous cows for higher milk supply, and the number of cows with Holstein Friesian blood levels is currently increasing. Higher milk yield in high-producing dairy cows, on the other hand, raises the cow's metabolic requirements, increasing the risk of metabolic diseases (Mekonnen *et al.*,2022).

Milk fever (hypocalcaemia) is one of the most common metabolic diseases caused by calcium metabolism problems that mostly affect high milk-yielding dairy cows around the world. Subclinical hypocalcaemia is more costly due to its higher prevalence and the likelihood of decreased productivity and reproduction, as well as increased reproductive and other diseases (Naher *et al.,* 2020). It affects a mature cow within 48 hours of giving birth, but it may occur several weeks before or after parturition. It is a non-infectious condition that affects dairy cows all around the world. It's also a common result of the modern dairy industry's aim for profit maximization (Bzuneh *et al.,* 2020).

Hypocalcaemia is considered as a gateway disease and predisposes the cow to various metabolic and infectious disorders in early lactation (Venjakob *et al.,* 2017). Subclinical hypocalcaemia is a well-described risk factor for reduced reproductive performance and increased risk of culling (Pinedo *et al.,* 2021). It reduced milk production, decrease immune function, slower uterine involution, delayed first ovulation after calving, reduced gastro-intestinal motility, increased risk of ketosis,decreased reproductive performance, retained placenta, mastitis, metritis, endometritis, displaced abomasum and increased risk of early removal from the herd (Naher *et al.,* 2020).

Milk fever affects dairy cows on sporadically, but it has recently become much more prevalent on small-scale dairy farms. Milk fever predisposes the cow to productivity problems, particularly in highly prolific dairy cows. It can be clinical or subclinical, depending on whether an animal shows clinical signs or not (da Silva *et al.*, 2019). Initial signs include ataxia, nervousness, and hyperactivity in the animal. Poor appetite, reduced rumen motility, low body temperature, sluggish breathing, impalpable pulse, weak but rapid heartbeats (80-100 per minute) that are difficult to hear due to impaired muscle contraction ability, dilated pupils, and dry muzzle are all common symptoms (Goff, 2008). Other signs and symptoms include a tilted head to the side, splayed out hind legs, and paresis (difficulty to rise from lying down). Finally, coma and sudden death are possible outcomes (Oetzel, 2012).

Calcium demand is relatively low during the dry season. As a result, intestinal calcium absorption and bone calcium reabsorption are generally inactive throughout this time. Lactation begins with a sudden loss of calcium through milk. Hypocalcaemia develops when the calcium homeostatic mechanism is unable to supply the calcium demand for milk production. After calving, blood calcium concentrations in about half of dairy cows in their second lactation and above fall below the threshold for subclinical hypocalcaemia (Bzuneh *et al.,* 2020). Calcium is a very important element for animals. It plays a great role in bone formation, muscular contraction, nerve transmission, blood coagulation, and many other basic biological processes in the body, as well as acting as a second messenger that regulates the effects of several hormones in animals (Adugna, 2019).

Close management of dry cow or management during late pregnancy and lactation is the keys to preventing milk fever (Sharma, 2015). Multiple techniques for managing hypocalcaemia and increasing calcium mobilization in dairy cows, particularly during the peri-parturient interval, through dietary management including: feeding anionic salts, low calcium ion diets, low potassium forages, and vitamin D supplementation (Amar, 2014). The most significant economic impact of milk fever is the decline in milk output, as well as the loss of animals owing to culling and mortality, as well as the cost of animal care (Thirunavukkarasu *et al.,* 2010).

Currently, researchers suggested that subclinical hypocalcaemia (SCH) is an important metabolic disorder that may predispose to clinical hypocalcaemia and in turn can be associated with the problems mentioned for clinical hypocalcaemia. Although clinical instances have a higher severity of disease, subclinical hypocalcaemia are also important, because they are significantly more common and incur significant economic losses. However, they are difficult to diagnose and many farmers are unaware of them (Mekonnen *et al.,* 2022). Subclinical hypocalcaemia is a major issue in Ethiopian dairy cattle. Recently, SCH was reported to affect 20.3% dairy cows in North-west Ethiopia and it was implied for reduced milk production with estimated financial loss of 11.25 Ethiopian Birr (ETB) and 3,026.25 ETB per cow per lactation, respectively (Mekonnen *et al.,* 2022). Although, there are large numbers of dairy farms in and around Bishoftu town, ample studies have not been conducted on metabolic disorders, especially on subclinical hypocalcaemia during transition period in dairy cow.

Therefore, the objectives of the study were:

- To determine the status of disease in the study area
- To identify the associated factors of the disease in study area
- To determine the serum calcium profile of dairy cows

2. LITERATURE REVIEW ON MILK FEVER

2.1. Etiology

Milk fever is a non-infectious dairy cow diseases that caused by a significant lack of metabolisable calcium ion (Ca^{2+}) in the blood (Oetzel, 2012). During onset of lactation, milk fever might develop because calcium is one of the most abundant minerals in cow's milk and the concentration of calcium in the colostrum is almost double than in milk later lactation of the cow (Tsioulpas *et al.*, 2007). Calcium from milk is frequently the primary source of calcium absorption. In a single milking, a cow producing 10 liters of colostrum loses about 23 grams of calcium (Kamiya *et al.*, 2010). As a result, the start of lactation puts a strain on a cow's ability to maintain calcium homeostasis. Colostrum and milk production increase around parturition, whereas dry matter intake falls temporarily, the result is a transient period of hypocalcaemia (Reinhardt *et al.*, 2011).

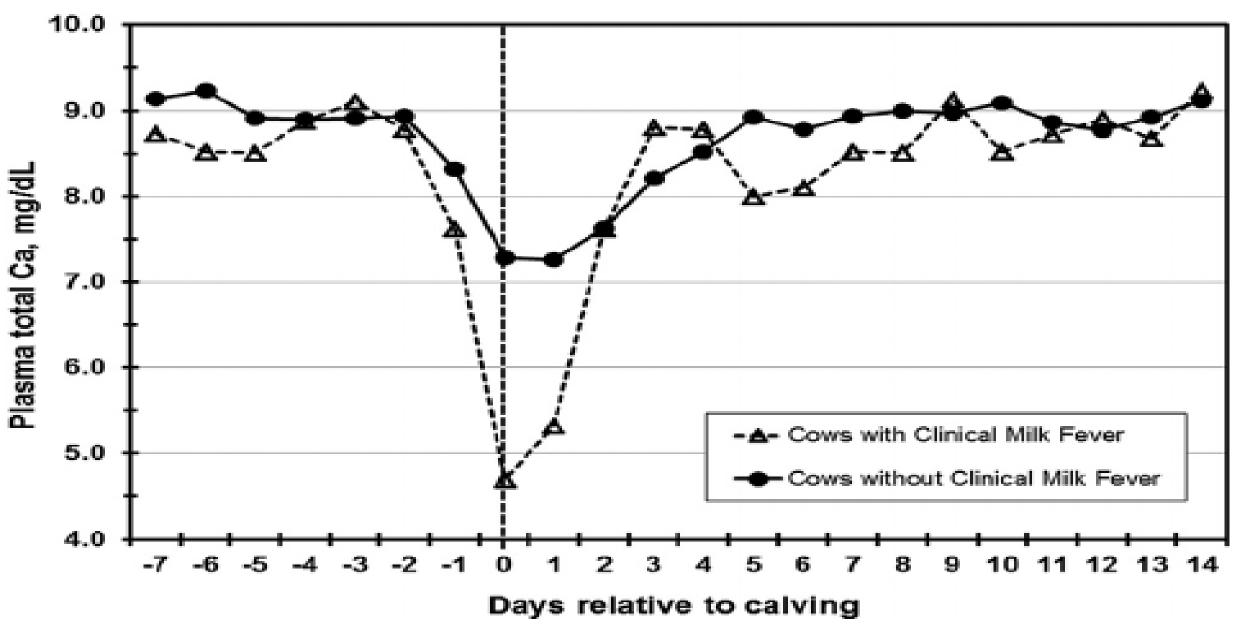


Figure 1: Total calcium plasma concentrations in dairy cows before and after calving

Source: (Kimura *et al.*, 2006)

2.1.1. Role of calcium in animal body

Calcium plays a critical role for immune cell function of animal and human being. A cows with hypocalcaemia are at an increased risk for developing immune-related infectious diseases such as mastitis and metritis (Rowson, 2011). It is the most component of bones and teeth. Calcium metabolism at calving (in cow) is one of the most vital animal wellbeing (health) components affecting the production, reproduction and feed change efficiency (Pandey, 2014). The variation in the relationship with health, production and reproduction may be due to differences in the metabolic and nutritional profiles and overall management of cows among dairy herds. Therefore, measuring a wide array of blood analytes may provide more insight into how circulating calcium concentration associate with cow performance during lactation (Gobikrushanth *et al.,* 2020).

As the body develops, bones are always shaped and transformed once more. To preserve the calcium levels, the calcium from bones will go into the blood when there's a low calcium level. Then the specified cells make new bone to replace the lost bone. As the bone is basically made up of calcium, when the body does not get sufficient calcium, the lost bone isn't replaced. As a result, without calcium, human creatures and animal (or any other living thing) would have no frame (Pandey, 2014). It is composed of a heterogeneous tissue that has the work of supporting the whole weight of the animal. Calcium also plays a critical part in the adjusting of cellular functions of nervous and muscular tissues, as well as of hormonal exercises and blood coagulati on (Abadia, 2019).

2.1.2. Calcium homeostasis

The mechanisms for calcium replenishment are relatively inactive during a cow's dry period, and the cow's intestine and bone adapt to lactation. When the homeostatic and homeorhetic mechanisms that control Ca metabolism not respond fast enough, clinical hypocalcaemia occurs. When the vitamin D cascade is triggered, vitamin D_3 (cholecalciferol), the inactive form of the vitamin is hydroxylated by 25-hydroxylases to the circulating form, 25-(OH) D_3 (calcidiol) in the liver, which is then hydroxylated by 1α-hydroxylase to the active form, 1,25-dihydoxyvitamin D_3 (calcitriol) in the kidney resulting in an increase in blood calcium (Golder *et al.,* 2021).

To avoid a decrease in blood calcium levels at the beginning of lactation, the animal should begin absorbing more calcium from the bones and diet to replace calcium lost in the milk (Ahad and Zaidi, 2016). In the absence of inflammation, parathyroid hormone and 1,25 dihydroxyvitam in D protect the extracellular pool by boosting intestinal absorption and renal reabsorption of calcium, as well as calcium and Phospharus bone resorption (Overton and Waldron, 2004).

The production of parathyroid hormone regulates the mobilization of calcium from the bone and is triggered by a drop in blood calcium level. 1, 25 dihydroxyvitamin D is another hormone which is necessary to increase intestinal absorption of calcium. Rise in blood parathyroid hormone level stimulate production of 1, 25 dihydroxyvitamin D from vitamin D and induces increase of intestinal calcium absorption. A variety of dietary variables disrupt Ca homeostasis, resulting hypocalcaemia. Parturition and hypocalcaemia leads to an immunosuppression through blunting of calcium signals in immune cells (Kimura *et al.,* 2006).

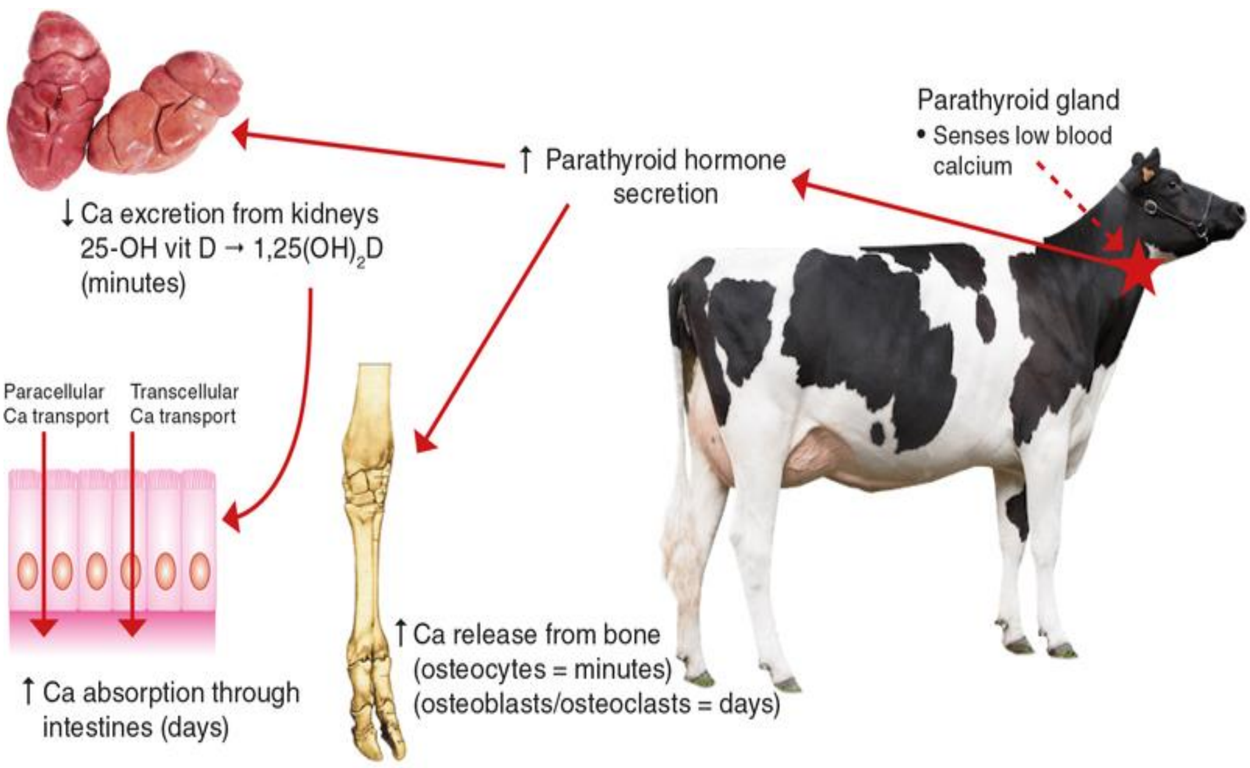


Figure 2: Homeostatic response of a cow to low blood calcium concentration

Source: Mcart, J. (2019)

2.2. Epidemiology

Milk fever is one of the common production diseases of dairy cows throughout the world. It occur sporadically in dairy cows, recently it is dramatically increased in small holder dairy farms. The occurrence of milk fever predisposes the cow to productive problems, especially among highly productive dairy cows (Fikadu *et al.*,2016). Milk fever was found to be substantially associated to milk output, parity and breed (Anteneh *et al.*, 2012).

Table1: Prevalence of milk fever in different part of Ethiopia

Authors	City	Prevalence
Adugna, 2019	Gondar	26.1%[SCH]
Akalu, 2017	Addis Ababa	27%
Anteneh *et al.*, 2012	Gondar	30.2%
Teferia *et al.*, 2016	Hawasa	17.5%
Tolosa *et al.*, 2021	Bale Robe	1.30%
Mekonnen *et al.*, 2022	Gondar	20.3%[SCH]

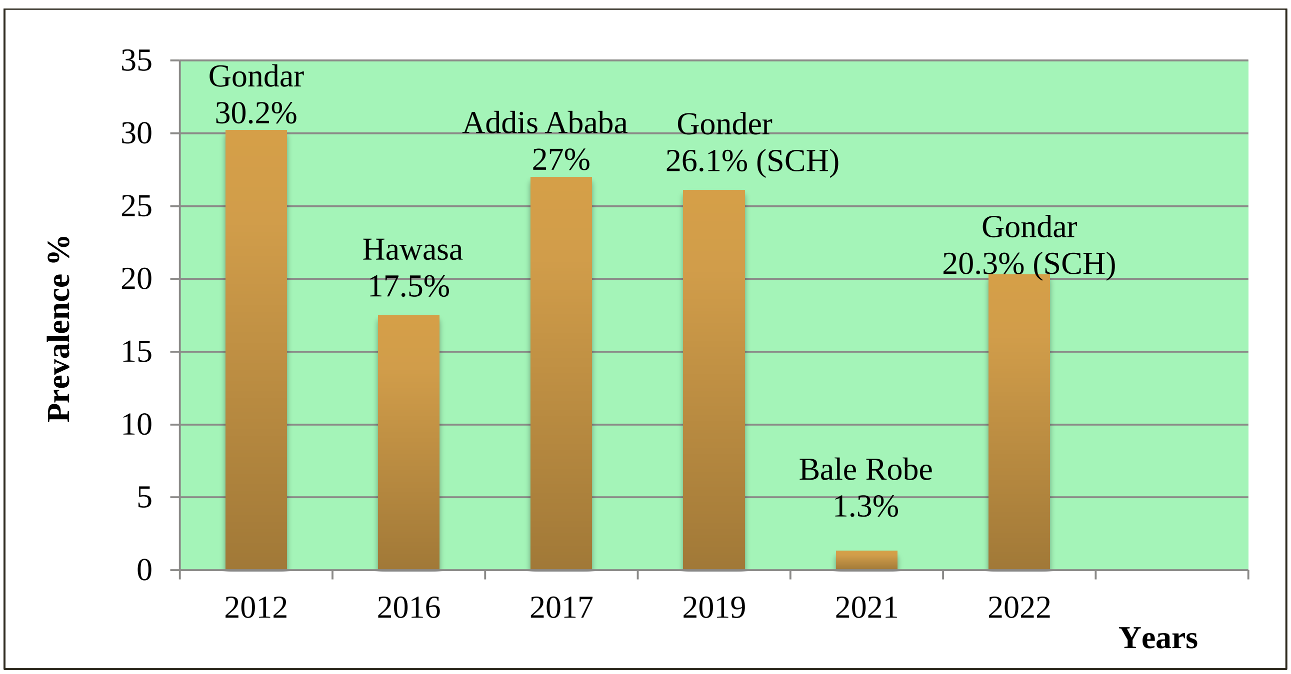


Figure 3: Bar graph representation on prevalence of hypocalcaemia is different years

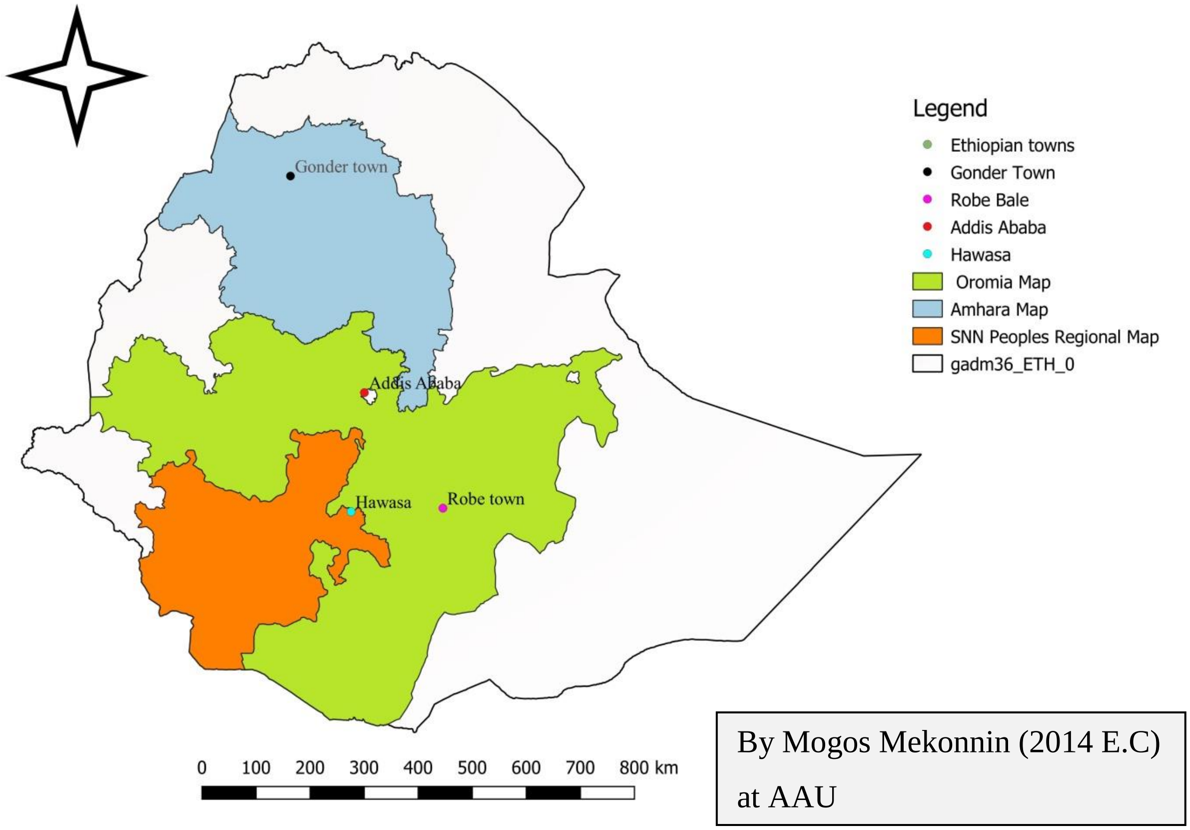


Figure 4: Prevalence distribution of milk fever in different part of Ethiopia

2.2.1. Risk factors

Predisposing factors that contribute to the occurrence of milk fever and which influence the prevalence and severity include age, stage of lactation and parity, body condition and diet composition, breed and length of dry period. In general, the risk factors for milk fever could be grouped into intrinsic risk factors, which are associated with the animal itself, and extrinsic risk factors, which are outside of the animal's body (Angassa, 2019).

Parity and milk yield: parity is one of intrinsic risk factor of milk fever and associated with high milk production at the cost of body condition and increased the occurrence of peri parturient disorders in dairy herds (Lee and Kim, 2006). Due to the significant osteoclastic activity in their growing skeleton, first-lactation dairy calves seldom get milk fever because they generate less colostrum and can rapidly mobilize calcium from bone (Ibrahim and Kirmani, 2021). Milk fever is associated with an increase in the number of calves born; hence parity is an essential risk factor for milk fever, with a 9 % greater risk for each subsequent lactation period (Taylor *et al.*, 2001).

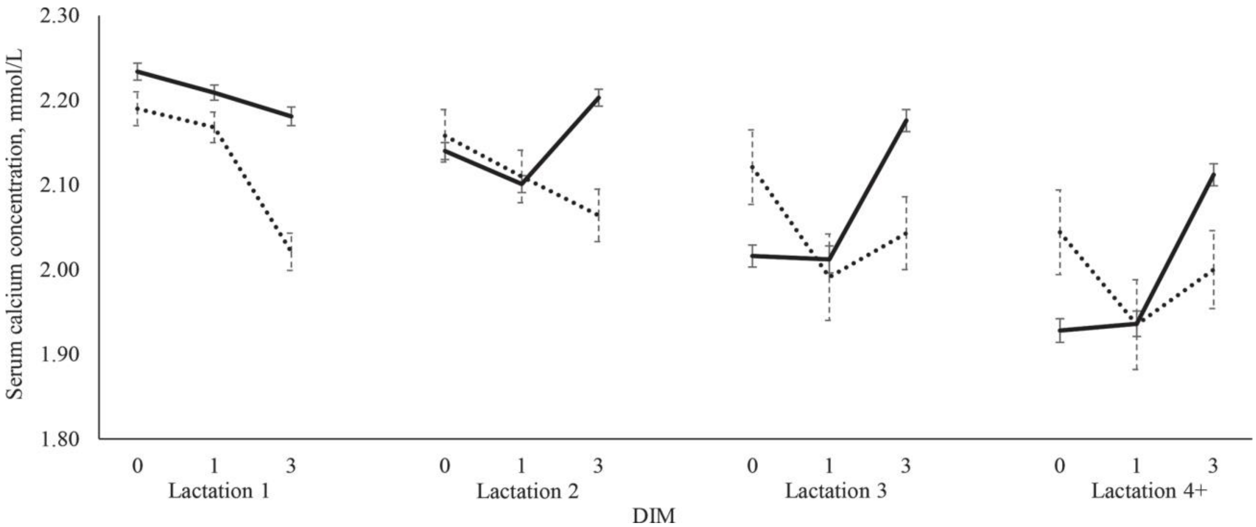


Figure 5: Association of serum calcium concentration with increasing parity of cow

Source: (Venjakob *et al.*, 2019)

Milk yield is an intrinsic risk factor for occurrence of parturient paresis. Since every liter of milk requires a certain amount of calcium, a larger production leads to a greater calcium loss, increasing the risk of milk fever (Daresj, 2020). The occurrence of milk fever is significantly associated with milk yield. Because, from a total of 116 cows affected by milk fever observed in Gondar town, 55 (50.92%) were producing 30 liters of milk per day. From local breeds only 6 (15%) had milk fever and from which 110 (31.9%) cross breed cows had the disease. This could be attributed to high milk yield of the cross breeds than the locals (Anteneh *et al.*, 2012).

Age of cow: is one of intrinsic risk factor that has a profound effect on exposure of dairy cows to hypocalcaemia and increases by approximately 9% per lactation (Naher *et al.,* 2020). Heifer's skeletal bones are still in the growth phase, thus they have a lot of osteoclasts, which can respond to PTH more quickly than adult cow skeletal bones (Ibrahim and Kirmani, 2021). Increased age also causes a decrease in the number of 1, 25-$(OH)_2D_3$ receptors (Rezac *et al.*, 2014). As a result, hypocalcaemia is age-related, peaking in cows between the third and seventh months of pregnancy. Research reports subclinical hypocalcaemia in postpartum cows increased with age and was present in 25% of first lactation cows and 41-54% of second or greater lactation cows (Bzuneh *et al.*, 2020).

Breed: it has been one of intrinsic risk factors that exposed dairy cows to metabolic diseases like hypocalcaemia. It's been shown that some dairy cow breeds are more prone to hypocalcaemia than others. Milk fever is more common in crossbred cows than in local breeds. This indicates that cows have a genetic predisposition to milk fever, which has been observed in some high-producing Jersey and other breeds (Tadesse and Belete, 2015). This is associated to increased milk production per unit of body weight and decreased vitamin D_3 receptors in the intestine (Peterson *et al.*, 2005). Intestinal receptors for 1, 25-$(OH)_2 D_3$ (1, 25 dihydroxy vitamin D_3) are lower in Jersey breeds than in Holsteins of the same age. Lower receptors would lead to a reduction of target tissue responsiviness and sensitivity to 1, 25$(OH)_2 D_3$, increasing the risk of hypocalcaemia (Weiss *et al.*, 2015).

The hypocalcaemic state of the dairy cow causes a rise in plasma 1, 25-$(OH)_2 D_3$ levels at calving. Normally, increased levels would result in increased bone calcium resorption and intestinal calcium absorption. However, because older animals have fewer receptors, the activation of genomic events by 1,25-$(OH)_2 D_3$ is insufficient, resulting in a greater tendency to become hypocalcaemic (Ibrahim and Kirmani, 2021).

Body condition score: is one of host- related risk factor of hypocalcaemia. Body condition score is based on the visual assessment and palpation of specific areas of the cow's body by the investigator (Vania and Plamen, 2016). The loin, ribs, tail head, brisket, flank, vulva and/or rectum and udder are the important parts of the body used in determining the score (Figure 6). It describes the systematic process of assessing the degree of fatness of an animal. The score reflects the plane of nutrition on which an animal has been exposed over a reasonable length of time. Physiologically, the proportion of protein and water of the animal's bodyweight decrease as it gains body condition (Ndlovu *et al.*, 2007).

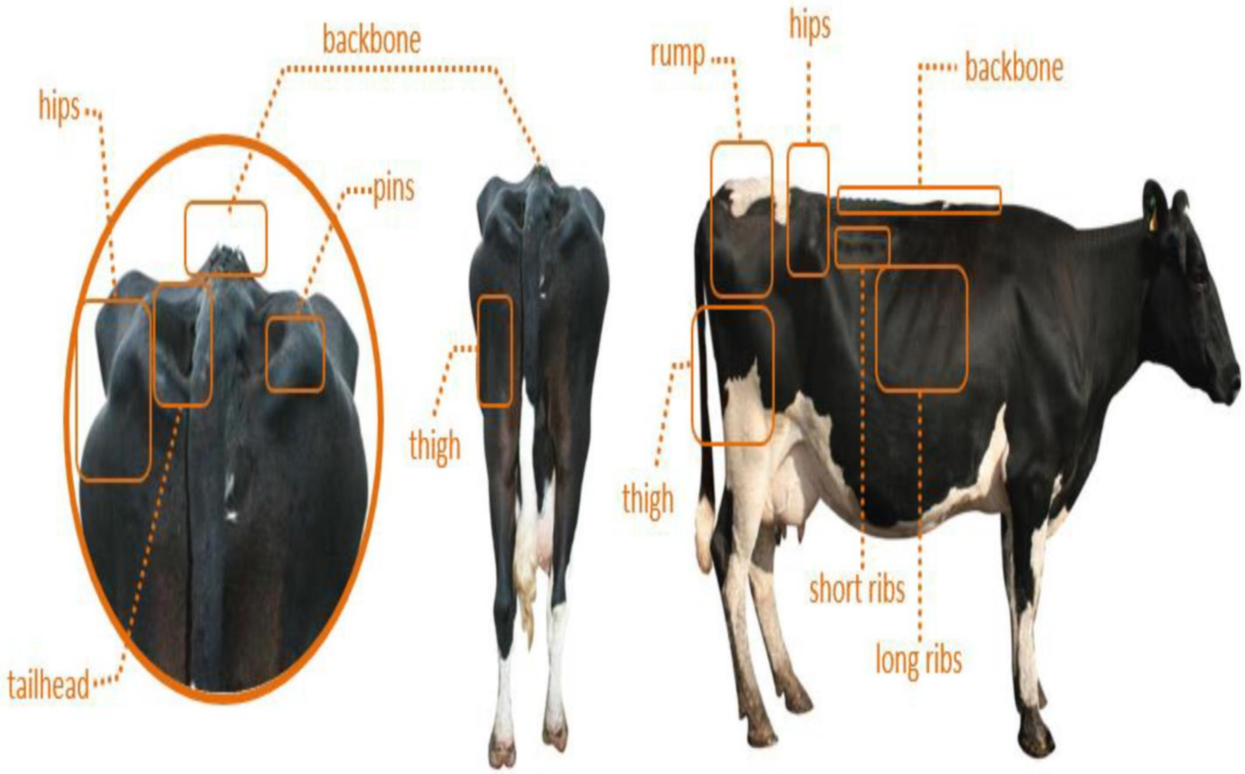


Figure 6: Key areas of cow body for body condition scores assessing.

Source: (Huang *et al*., 2019)

Milk fever is four times more frequent in dairy cows that are over conditioned at calving (Ostergaard *et al.*, 2003). This is because dairy cows with a higher body condition score at calving produce more calcium in their milk, making them more susceptible to milk fever, and over conditioning causes decreased feed intake throughout the gestation period since they only eat a limited amount of calcium-containing feed. This is due to a decrease in appetite during the key period around calving (Harris *et al*., 1999).

Body condition scoring was based on the criteria: a) In BCS 1 (very poor body condition) individual short ribs have a thin covering of flesh, b) In BCS 2 (poor body condition), individual short ribs can be felt but are not prominent, c) In BCS 3 (good body condition), ends of short ribs can be felt by applying slight pressure, d) In BCS 4 (fat), individual short ribs are distinguishable only by firm palpation, e) In BCS 5 (very fat), bony structures of backbone, short ribs, and hook and pin bones are not apparent (Abunna *et al.*, 2018).

Feed factor: is one of extrinsic risk factor of hypocalcaemia**.** Feeds that provide a high daily calcium intake to dry cows have been associated to an increased prevalence of parturient paresis. Because active absorption of dietary calcium and bone reabsorption are reduced at this level, the calcium maintenance requirement can be satisfied mostly through passive absorption. Cows in this disease are unable to restore the calcium lost in milk in their plasma and become severely hypocalcemic (Boda and Cole, 2003). Higher calcium intake impairs the uptake of calcium. Prepartum diets high in cations like sodium and potassium are associated with an increased incidence of milk fever (Bhanugopan and Lievaart, 2014).

Table 2: Nutritional factors responsible for milk fever

Factor	**Effect**
General Nutritional status at prepartum period	Increase K pasture, over fat cow and very thin Cows
Reduced feed intake on day of calving	Reduce calcium intake and absorption
Increase Ca intake prepartum	Increase passive absorption and quiescence of parathyroid gland
Increase P intake prepartum(>80 g P/d)	Inhibits vitamin D metabolism
Reduce Mg concentration at prepartum	Reduce production and secretion of PTH
Dietary Cation Anion Differences	Increase DCAD balance

Source: (Patel *et al.,* 2011)

Complete milking: is one of extrinsic risk factor that predispose dairy cow to hypocalcaemia. It is an absolute withdrawal of milk from the cow at milking time. Complete milking within the first 48 hours (two to three days) following calving appears to be a factor in the development of milk fever. When the cow has finished milking, it will immediately begin secreting milk again. As the cow exports a substantial amount of calcium into the milk, this results in an equivalent loss of calcium from the body. Therefore, complete milking, especially in vulnerable cows, can thus predispose the animal to milk fever (Eslamizad *et al.,* 2010).

2.3. Transition Period of Dairy Cow

Transition period is defined as the three (3) weeks leading up to the estimated date of delivery (EDD) and the three (3) weeks following parturition. It is a period when cows experience considerable metabolic changes in glucose, fatty acids, vitamins, and mineral metabolism in order to prepare for the arrival of the fetus and the start of lactation (Abdelmenem *et al.*, 2020). Metabolic disorders and health problems are common during this time and can easily erase the entire profit potential for dairy cow farms. Within this interval, the practical purpose of dietary management is to support the respective metabolic adaption (Simenew and Wondu, 2013).

When health problems arise during the transition period, milk production is lost for the duration of the sickness, and typically for the entire lactation. The cow's profitability during that lactation is largely determined by the success of the changeover period. During this phase, a cow's capacity to reach maximum milk output may be hampered by nutritional or management constraints (Drackley, 1999). Increased energy and nutrient demands for colostrum and milk synthesis, combined with decreased feed intake, cause negative energy balance (NEB) and micronutrient deficits in transition cows (Wankhade *et al.*, 2017; Barraclough *et al.*, 2020).

2.3.1. Lactating dairy cow

The initiation of milk production challenges the ability of the lactating dairy cow to maintain normal calcium concentrations. Once lactation begins, blood calcium levels drop leading to mobilization of calcium reserves from skeletal stores (Shock *et al.*, 2019). Lactation evolved to be the core functional system of providing maternal care in mammalian species. The mammary gland provides an ideally composed nutrient source for the newborn. Mainly in early lactation, most of the available nutrients are directed to the mammary gland, both those from feed, and those mobilized from body tissue. Therefore, milk production can be maintained at a high level despite a negative energy balance (Bruckmaier and Gross, 2017).

There are main stages in the lactation cycle of the dairy cow; Early lactation (14-100 days), mid lactation (100 to 200 days), late lactation (200-305 days). At early lactation, cows will achieve peak milk production (during the second month of lactation for Holstein cows), feed intake is lagging and cows are usually losing weight. At the end of early lactation, peak dry matter will be achieve and no weight losses occurring. During this period milk yield increases more rapidly than dry matter intake (peak production) and more calcium (Ca) drawn into milk production. The demand for energy is therefore higher than the amount of energy consumed. Thus the cow mobilizes body reserves and losses weight (negative energy balance)(Emmick, 2000 ; McNamara and Shields, 2013).

Negative energy balance has been associated with compromised immune and reproductive functions in dairy cows. The most common clinical diseases in dairy cattle are metritis and mastitis, both of which have been negatively associated with subsequent reproductive performance. In addition, dairy cows develop the so called subclinical disorders, such as subclinical ketosis and hypocalcaemia (Santos and Ribeiro, 2014). The requirement for absorbed calcium is the sum of the individual requirements for maintenance, growth, pregnancy, and lactation. The concentration of dietary calcium required is dependent on the availability of calcium from the feed and the efficiency of intestinal absorption (Taylor, 2007).

2.3.2. Pregnant dairy cow

In dairy cattle, the late pregnancy period is important to prepare the mammary gland for lacta tion and drain high amount of calcium to mammary site for colostrum production. This is also a period of utmost importance for fetal development (Sguizzato *et al.*, 2020). Deficient care during late pregnancy can result in decreased colostrum yield and quality, diminished milk yield and component concentration and increased incidence of health disorders. In actuality, the late pregnant cow is not resting at all. Therefore, the late pregnancy period should be viewed as a lactation preparation period, thus emphasizing the critical role of effective management on subsequent lactation performance (Van Saun and Sniffen, 1996). Late pregnancy nutritional requirements and management practices implemented during the last 3 to 4 weeks necessary to optimize lactation, health and reproductive performance of cows (Beede *et al.*, 1991).

2.4. Clinical Manifestation

The calcium drainage to the mammary gland along with the reduced capacity of the animal to mobilize calcium from bone reserve lead to a drop of the calcium blood level with paresis like clinical symptoms known as milk fever (Masoero *et al.*,2003). Depend on the degree of hypocalcaemia and time of occurrence, the clinical signs of milk fever divided into three stages (Figure 7). Stage I is characterized by mild excitement and tetany without recumbency. This phase often goes unobserved because its signs are subtle and of short duration (often less than 1 hour) (Fikadu *et al.*, 2016).

Excited, nervous, anorexia, weakness, weight shifting, and shuffling of the hind leg are all symptoms of an affected cow. Stage II milk fever causes sternal recumbency in cows. They have moderate to severe depression, cold extremities, slight bloating, and partial paralysis, and they usually sleep with their head twisted to the side (Oetzel, 2012). Stage III hypocalcaemia cows are fully paralyzed, swollen, in lateral recumbency, gradually lose consciousness and eventually resulting in coma. There is a significant drop in temperature, an increase in heart rate, and animals will die if not handled/treated (Fekata, 2021).

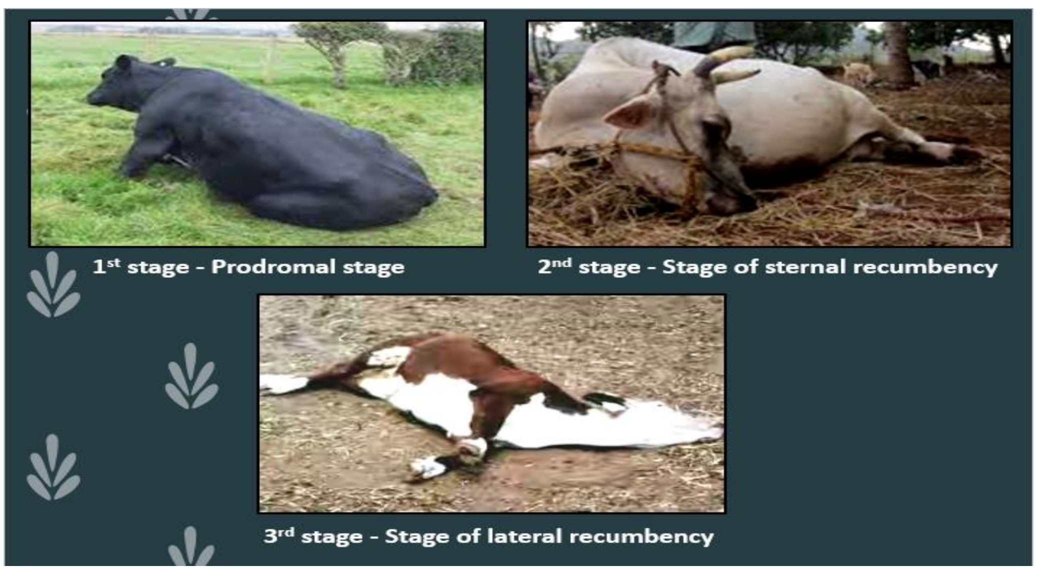


Figure 7: Dairy cows that are in the various clinical stages of milk fever (hypocalcaemia)

Source: ***http://veterinarysciencehub.com/clinical-signs-of-milk-fever/***

2.5. Differential Diagnosis

To correctly diagnose milk fever, differential diagnosis is required to distinguish it from other illnesses with similar clinical indications, such as hypoglycemia. Rapid progression of the disease with mortality within 6 to 12 hours in hypocalcaemia and a slow course of the disease with death within 5 to 7 days in hypoglycemia are typical signs and indicators that distinguish milk fever from hypoglycemia. Hypoglycemia induces chin elevation (star gazing posture) with a 2 to 3 day delay in progressing to recumbence after the start of early symptoms, whereas hypocalcaemia causes rapid recumbences over 3 to 4 hours with sternal recumbence (Tadesse and Belete, 2015). Most cases can be differentiated from milk fever, as hypocalcaemia has a rapid response and good recovery to administration of IV calcium borogluconate (Bewley and Schutz, 2008).

2.6. Diagnosis of Milk Fever

Hypocalcaemia is diagnosed using a combination of history collection, clinical examination, and laboratory testing. During the history, all detailed information about the cow should be obtained, including age, breed, lactation stage, milk yield, and calving day (Fekata, 2021). Milk fever is a disease that affects mature dairy cows between the ages of 5 and 9 years old, usually within 72 hours of giving birth. The most accurate approach of diagnosing a case of milk fever is laboratory assessment of blood calcium levels for subclinical hypocalcaemia and satisfactory response to intravenous calcium solutions for clinical hypocalcaemia (Bzuneh *et al.,* 2020).

The measurement of specific metabolites allows the evaluation of adequacy of the main metabolic pathways associated with energy, protein, and minerals and then provides useful information related to nutrition and animal health to optimize the productive and reproductive potential of cows (Morales *et al.*, 2016). The normal serum Ca concentration is 8.5-10 mg/dL (2.15-2.5 mmol/L) (Goff, 2008). Animals with serum calcium level of 5.5 to 7.5 mg/dL show sign of stage I hypocalcaemia. Stage II hypocalcaemia seen with calcium levels of 3.5 to 6.5 mg/dL and stage III seen when calcium concentration falls below 3.0 mg/dL (Bzuneh *et al.*, 2020).

2.7. Physiological Effect of Hypocalcaemia in Dairy Cow

The influence of hypocalcaemia on dairy cattle welfare and production extend beyond its clinical symptoms. Inadequate concentrations of ionized Ca in the circulation impaired immune cell function and smooth muscle contraction (Hansen *et al.*, 2003; Kimura *et al.*, 2006). It has been associated with physiological disorders affecting the contraction of calcium-dependent smooth muscle and skeletal muscle. It has been associated with a higher incidence of uterine prolapse and retained fetal membranes, because uterine involution is less efficient in cows with hypocalcaemia (Risco *et al.*, 1994).

Cows with milk fever have abnormally long intervals between calving and first postpartum ovulation, and a longer luteal phase in the subsequent estrus cycle. Subclinical hypocalcaemia was characterized as a postpartum circulating concentration of Ca <2.15 mmol/ L (Macmillan *et al.*, 2020). It affects about 50% of all multiparous cows and 25% of heifers (Reinhardt *et al.*, 2011). It occurs in some high producing dairy cows during the first 40 days after a normal calving, reduces the size of the ovulatory follicles and their associated corpora lutea when the normal cycle is resumed after calving (Murray *et al.*, 2008).

Physiologically, low plasma concentrations of Ca^{2+} have been associated with low intracellular Ca^{2+} in polymorph nuclear inflammatory cells, which may decrease phagocytosis and increase the susceptibility of cows with hypocalcaemia to infections such as mastitis and metritis after calving (Ducusin *et al.*, 2003). There is a strong association between low calcium levels and decreased immunological function, which can lead to infectious illness. In hypocalcemic cows, a low calcium level lowered muscle tone in the teat end sphincters. A low calcium level decreases a cow's natural immunity, increasing the likelihood/chance of mastitis-causing bacteria infecting the cow (Mekonnen *et al.*, 2022). Generally, schematic view of impact of hypocalcaemia in dairy cows indicated in (Figure 8).

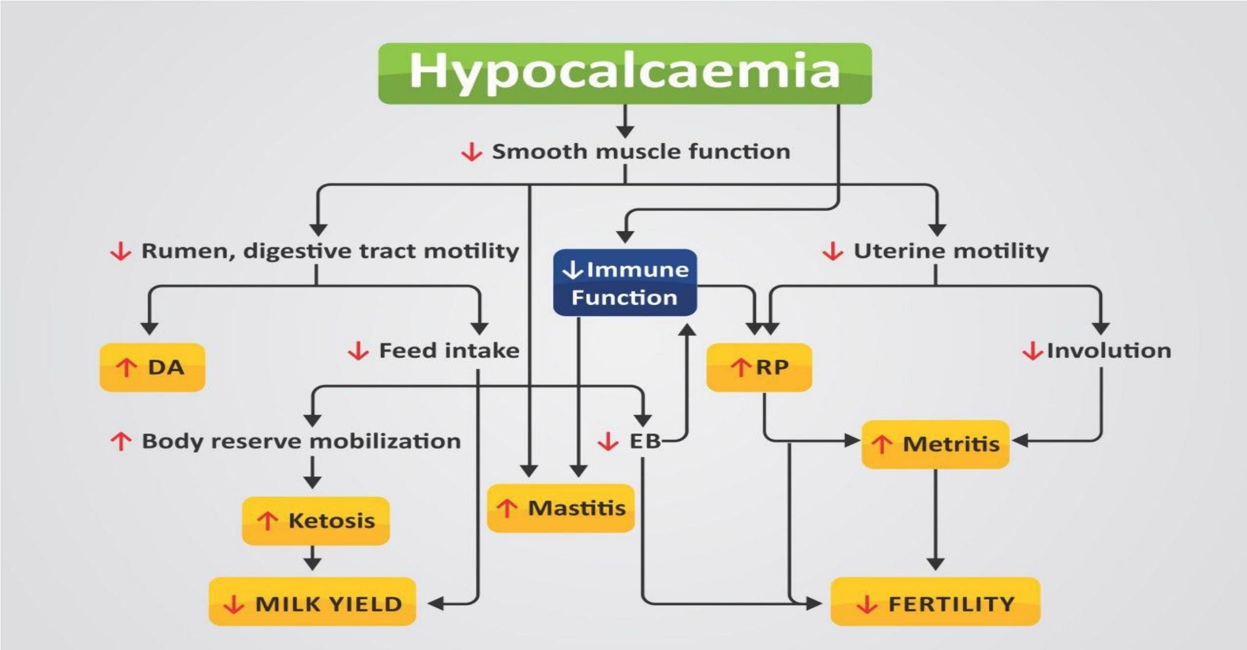


Figure 8: Schematic view of impact of hypocalcaemia in dairy cows

Source: (Seifi and Kia, 2018)

2.8. Pathogenesis of Milk Fever

The key component for the onset of milk fever is an imbalance between Ca production in milk and calcium inflow to the extracellular pool from bone and intestine (Mcguffey, 2017). In adult cows the normal peripheral blood Ca^{2+} concentrations are in the range 8.5 to 10 mg/dl. The entire extracellular pool contains only 8 to 9 g of calcium, a third of which is in the plasma pool (Murray *et al.,* 2008). There is a significant loss of calcium in cow since high demand for calcium in the body of cow and blood calcium is decreased at the second or the third day of after calving cow (Rodríguez *et al*., 2017).

Because of the loss of blood calcium to milk might exceed 50 g per day, changes in calcium metabolism generated by lactation are more important than parturition per se in the pathogenesis of parturient paresis. Before calving, the daily Ca requirement is only 30 g, with 15 g lost through faecal and urine waste and 15g going to fetal growth (Sundrum, 2015). This demand for calcium may only be satisfied by increasing absorption from the rumen or intestines, and increasing mobilization from tissue, especially bone reserves of calcium, as circulating blood calcium reserves are limited. If calcium concentration is low in the circulation, animals may develop hypocalcaemia (DeGaris and Lean, 2008).

The mobilization of bone calcium is regulated by parathyroid hormone (PTH), secreted whenever there is a decrease in plasma calcium ion. Several factors can inhibit bone resorption: oestrogen, the product of placental steroidogenesis during the period immediately before calving; calcitonin, released by thyroid Para follicular C-cells in response to hypercalcaemia, which also increases the urinary excretion of calcium and age, because older cows have fewer active osteoclasts capable of responding to PTH (Murray *et al.*, 2008).

Milk fever has three main effects on muscle contractions. Firstly, calcium has a membrane stabilizing effect on the peripheral nerves. Hyperesthesia and mild tetany seen in early stages of milk fever are due to lack of nerve cell membrane stabilization. Secondly, calcium is required for the release of acetylcholine at the neuromuscular junction. The inability to release acetylcholine, due to hypocalcaemia causes paralysis by blocking the transmission of nerve impulse to the muscle fibers. Thirdly, muscle cells require calcium directly for contraction. The clinical indications of parturient paresis are caused by the paralysis of several muscle groups (Ibrahim and Kirmani, 2021). Currently it is known that the pathogenesis of the disease is much more associated with the action of PTH on cells responsible for demineralization (osteoclasts), cells of the intestine responsible for absorption and kidney cells responsible for the reabsorption of calcium in the tubules (Bezerra *et al.*, 2014).

2.9. Treatment of Milk Fever

Treatment of milk fever must be done as soon as possible with the aim to restore the serum calcium concentration to avoid muscle and nerve injury (Firdaus *et al.*, 2016). Its treatment as application of Calcium and clinical response is also being considered as satisfactory based on the stage of the disease. Intravenous administration of calcium borogluconate or subcutaneous injection with 1.5g of calcium is the common practiced in treatment of milk fever in dairy cows (Thilsing-Hansen *et al.*,2002; Sasaki *et al.*, 2013; Miltenburg *et al.*, 2016).

Supplementation of calcium borogluconate by oral route is the best option to hypocalcemic cows that are still standing, such as cows in stage I hypocalcaemia or which have undetected subclinical hypocalcaemia. For cows in stages II and III of milk fever, a slow IV infusion of 500 mL of 23 percent calcium borogluconate should be given immediately. Extremely high dose of calcium may cause fatal cardiac complications. Subcutaneous calcium administration can also be used to support blood calcium concentrations around calving (Bzuneh *et al.,* 2020). Moreover, addition of magnesium in the solution containing calcium borogluconate will help in inducing parathyroid hormone receptors to stimulate calcium absorption (Firdaus *et al.*, 2016).

2.9.1. Effect of calcium supplementation on hypocalcaemia

Clinical and subclinical hypocalcaemia are common issues in the dairy industry and both are associated with a variety of negative sequel including an increased risk of displaced abomasum, mastitis, retained placenta, dystocia, and ketosis (Shock *et al.,* 2019). Treatment with a calcium-containing bolus post calving was associated with a lower incidence of subclinical hypocalcaemia, improved pregnancy per breeding, shorter days to pregnancy, and higher milk production in multiparous cows (Martinez *et al.,* 2016). Supportive treatment with electrolytes, vitamin and glucose are also recommended and practiced for the better prognosis. Anti-inflammatory and glucocorticoids are also recommended to minimize tissue injuries and prevention of stress factors (Mar, 2021).

2.10. Prevention and Control of Milk Fever

Milk fever prevention is strategically significant for dairy farmers, because it reduces production loss, death loss, and veterinary costs associated with the disease. Milk fever could be managed carefully, since it is a gateway illness that drastically reduces the chances of dairy cows being fully productive and reproducing. In the dairy industry, a specific management approach is necessary to reduce the occurrence of milk fever among high-risk cows (Fekata, 2021).

Several techniques have been used to prevent hypocalcaemia and mobilize Ca^{2+} in dairy cows through dietary management including feeding anionic salts, low calcium ion diets, low potassium forages, and vitamin D supplementation (Amar, 2014). Hypocalcemic cows have decreased feed intake and rumination, increased plasma concentrations of cortisol. Oral supplementation of calcium (Ca) has been considered as a complement to dietary strategies to manage hypocalcaemia in dairy herds. It benefited reproduction in multiparous cows and milk-yield in the cohort of multiparous cows of higher milk production potential, but it was detrimental to reproduction in primiparous cows (Pinedo *et al.,* 2021).

The ultimate goal of oral Ca administration is to provide a large quantity of Ca that is absorbed over a longer period, resulting in a steady and prolonged increase in blood Ca concentration compared with injectable products. These products contain differing combinations of rapidly and slowly absorbed Ca salts. The benefit of rapidly absorbed salts, such as Ca chloride, is its highly bioavailable and acidifying (supporting mobilization) of the cow’s own Ca stores. Slowly absorbed Ca salts (such as Ca propionate, Ca sulfate, and Ca carbonate) either have an equivalent efficacy and longer duration of action than quickly absorbed Ca salts (Ca propionate) or are ineffective as an immediate Ca source due to their poor bioavailability (Wilkens *et al.,* 2020).

In current commercial dairy farms, many milk fever control principles and elements are used. Oral drenching with an easily absorbed calcium supplement around calving, feeding acidifying rations with anionic salt supplementation during the last weeks of pregnancy, feeding low calcium rations during the last weeks of pregnancy, and management practices such as body condition control and shortening the dry period (Radostits *et al.,*2007). Control of milk fever has revolved around stimulation of homeostatic mechanisms through feeding a pre-calving diet low in calcium (DeGaris and Lean, 2008).

It is critical to ensure appropriate magnesium supplementation in order to avoid milk fever. Because magnesium (Mg) is a crucial intermediary in the reabsorption of calcium from bone by parathyroid hormone, it is particularly important in Ca metabolism (PTH). Increased Mg supplementation was reported to have the strongest impact among dietary interventions for milk fever prevention (Ibrahim and Kirmani, 2021). Magnesium (Mg) is the body's second most abundant intracellular element. It is critical to the nerve transmission mechanism. It's also necessary for the parathyroid hormone's regular generation and action. As a result, it is considered as a chief player in the metabolic profile of dairy cows (Kronqvist *et al.*, 2011).

3. ECONOMIC IMPORTANCE OF MILK FEVER

The art of feeding dairy cattle is rapidly becoming the basic and applied science of dairy cattle nutrition. Milk fever is a metabolic disease that affects high-producing dairy animals within one or two days of calving, resulting in a significant decrease in milk output and thus becoming economically crucial (Patel *et al.,* 2011). The reproductive capability of the cow, a major concern in its economic value in dairy farming, is frequently related to periparturient events, as undesirable health related events during this period might result in tremendous economic losses to farmers (Thirunavukkarasu *et al.,*2010). Milk fever is associated with mastitis and endometriti s and causes reductions in milk yield and quality that result in substantial economic losses to the dairy industry (Sun *et al.,* 2014).

3.1. Direct Impacts of Hypocalcaemia

Because of the disease's tremendous economic effects, milk fever is a serious concern in many countries. Losses due to reduced milk output of infected cows, loss of animals through death and culling, and the expense of treating the animals are the most significant direct economic losses caused by milk fever (Thirunavukkarasu *et al.,*2010). As different research indicated that, cows with metabolic diseases produced less milk than healthy cows (Paiano *et al.,* 2019). Recently, SCH was reported to affect 20.3% dairy cows in North-west Ethiopia and it was implied for reduced milk production with estimated financial loss of 11.25 Ethiopian Birr (ETB) and 3,026.25 ETB per cow per lactation, respectively (Mekonnen *et al.,* 2022).

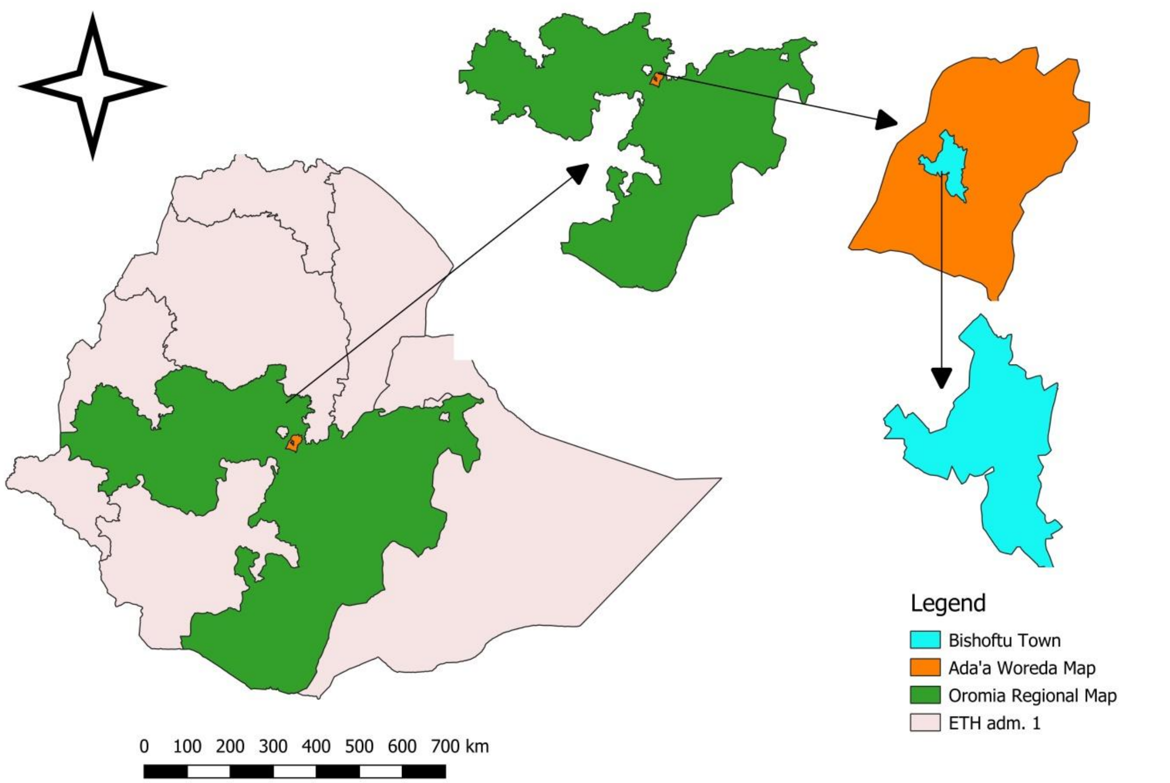
3.2. Indirect Impacts of Hypocalcaemia

It predisposes dairy cows to a variety of health problems, increasing dairy farmers' costs indirectly through cow deaths, culling, treatment, and management regimens. The indirect cost of milk fever is owing to an increased risk of linked health problems, calving problems, and the possibility of death (Bzuneh *et al.,* 2020). Hypocalcaemia in the subclinical stage reduce the ability of the transition cow to effect smooth and skeletal muscle contraction (Goff, 2004). Cows with hypocalcaemia have less muscle contraction, which reduces the motility of their digestive organs, such as the abomasum and the rumen (Paiano *et al.*, 2019). This reduction in ruminal and abomasal motility will likely cause a reduction in feed intake (Whiteford and Sheldon, 2005).

4. MATERIALS AND METHODOLOGY

4.1. Description of the Study Area

The study was conducted in and around Bishoftu town eastern part of Ethiopia. Bishoftu is a town located in the Ada'a district, East Shewa zone at a distance of 45 km South East of Addis Ababa, Ethiopia. The town is located in east Shewa zone of Oromia region and it lies 9˚ North latitude and 40˚ East longitude at an altitude of 1850 meters above sea level in the central high land of Ethiopia. It has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September) and the remaining in the short rainy season extending from March to May. The mean annual maximum and minimum temperatures of the area are 26˚C and 14˚C, respectively, with mean relative humidity of 61.3% (Berhe *et al.*, 2019).



By Mogos Mekonnin (2014 E.C) at AAU

Figure 9: Map of study area

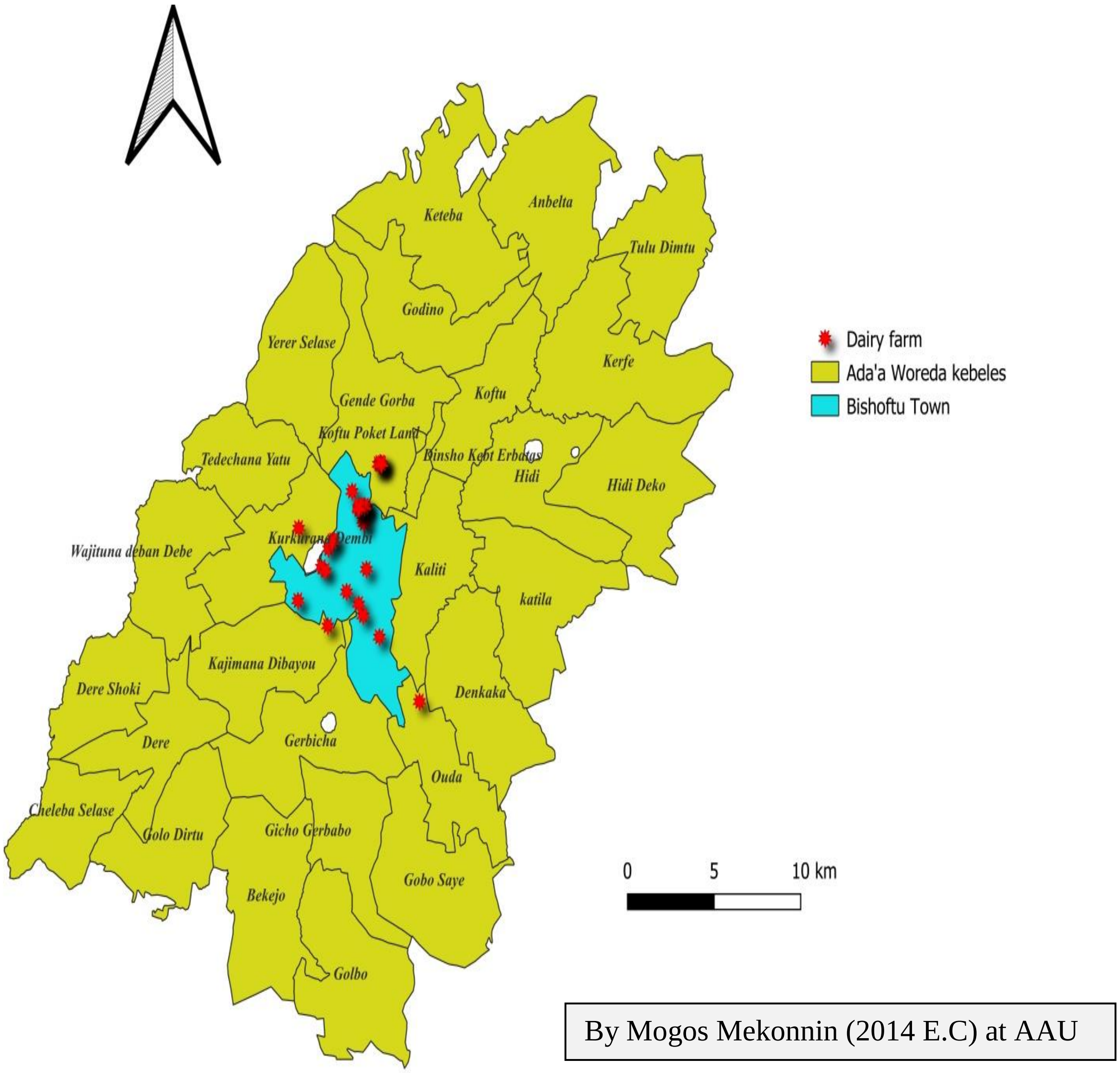


Figure 10: Geographical location of sampled dairy farm in study area

4.2. Study Population and Sample Size Determination

This study was conducted on dairy cows owned by private and some government in and around Bishoftu town. The majority of dairy producers in the town are market oriented smallholder dairy farmers with average herd size of three (3) cows which are organized under, one dairy cooperative called, Ada’a milk and milk products marketing cooperative share company.

A sampling frame: the list of current members of Ada'a Milk and Milk Products Marketing Cooperative was acquired from the administrative office, at the starting of study. A list of 115 farms housing dairy animals were considered as the sampling frame.

For an item scored 0/1 for no/yes, the standard deviation of the item scores is given by $SD = [p(1-p)/N]^{1/2}$ where p is the proportion obtaining a score of 1, and N is the sample size. The standard error of estimate SE (the standard deviation of the range of possible p values based on the pilot sample estimate) is given by $SE = SD/N^{½}$. Thus, SE is at a maximum when p = 0.5. Thus the worst case scenario occurs when 50% agree, 50% disagree. The sample size, N, can then be expressed as largest integer less than or equal to $0.25/SE^2$. Thus, for SE to be 0.01 (i.e. 1%), a sample size of 2500 would be needed; 2%, 625; 3%, 278; 4%, 156, 5%, 100. $N=0.25/SE^2$, where, N= sample size, SE=Standard error of the population (Arsham 2007).

But, because of unwillingness of some farm owners, only 43 dairy farms (Small-scale, Medium scales, and Large-commercial scales) were selected from the cooperation. Among the selected farm, 39 dairy farms were private whereas 4 dairy farms were government farms.

4.2.1. Sampling method

The selection of the herds or dairy farm was done by purposive sampling and based on the farmers' willingness to participate in the study and the presence of late pregnant cow and/or cow at early lactation stage in the herd. Accordingly, a total numbers of 176 dairy cows were sampled, based on the number of animal in the farm during the study period.

4.2.2. Study animals

Private dairy cows, as well as some government-owned Cross Holstein Friesian and local zebu breed dairy cows, were used in the study. Among dairy cows examined, 132 of cows were raised under intensive management and 44 cows were managed by the semi- intensive system. The farms were categorized into defined strata based on herd size. Therefore, Small scale (farms with less than 3 cows), Medium scale (farms with 3-10 cows) and Large scale (farms with greater than 10 cows) as suggested by ILRI, (1996).

4.3. Study Design

A cross-sectional study was carried out from November, 2021 to June, 2022. Data were generated using questionnaire survey and blood sample collection.

4.3.1. Questionnaire survey

Pre-tested semi structured questionnaires were administrated through personal interview to farm owners and attendants of all farms considered for the study. The questionnaire has closed and an open ended question that includes animal feeding, production status per day, and history of milk fever occurrence, history of retained placenta, management systems and parity (Appendix 2). Participants were informed of the study objective and their consent was obtained before interview. Animal data were collected using standard sheet (Appendix 1). Data on each animal such as breed, body condition, parity, production status, and management, feed type and age were obtained through direct observation and through questionnaire survey of owner at the time of sample collection.

4.4. Sample Collection and Laboratory Test

4.4.1. Blood sample collection

After the cows were properly restrained, a blood sample of about 10ml was taken from the jugular vein using plain vacutainer tubes. Within 5 hours of sampling, blood samples were stored in a vertical rack and delivered to the laboratory. The samples were properly labeled and stored in the icebox and transported to the Addis Ababa University, College of Veterinary Medicine and Agriculture, Microbiology laboratory for serum separation. Blood samples were centrifuged at 3000 rpm for 15 minutes. Then, serum was separated and transferred into crovials tube by using plastic pipette and maintained at -20°C until serum calcium levels were tested at Adama Public Health Research and Referral Laboratory Center (APHRLC).

4.4.2. Serum calcium analysis

Serum was utilizing for biochemical analysis, which total serum calcium concentration was subjected to analysis employing Cobas 6000 electrolyte analyzer. The steps of analyzing were adapted from manufactures (Roche diagnostic company, Germany). Serum calcium was measured by using Cobas 6000 serum electrolyte analyzer. Cows with serum calcium of < 2.15mmol/L were considered subclinical hypocalcaemia (SCH) otherwise free of subclinical hypocalcaemia (Martinez *et al.*, 2012).

4.5. Data Management and Analysis

All the epidemiological research findings were stored in Microsoft Excel and analyzed using the R studio software, version 4.0.3 (2020-10-10). The QGIS desktop version 3.20.0 was used only to show the geographic location of dairy farms sampled in the study period, the study area and some previous prevalence of milk fever in different parts of Ethiopia. Descriptive statistics were used to describe the nature and characteristics of the data. The prevalence was calculated by dividing the number of positive samples (that is, when the serum calcium level of a sampled cow fell below the cut-off point (2.15mmol/L), we were considered to have subclinical hypocalcaemia, and when the serum calcium level of a sampled cow fell between the normal range (2.15-2.5 mmol/L), we were considered to have normocalcaemia) by the total number of serum samples tested.

The difference between the effects of some associated risk factors and subclinical hypocalcaemia was analyzed by a Chi square test (Table 4). A multiple linear regression model was also performed to determine the association between serum calcium concentration and its risk factors (Table 6). Multiple linear regressions were conducted by considering all variables (Model 1). Then, we considered the factor that had a significant impact on the variable of interest i.e., $p < 0.05$. Next, a multiple linear regression reduced (Model 2) was performed (with a $p<0.05$). The next step we performed was ANOVA of both multiple regressions (model containing all variables) and multiple reduced (model containing significant variables). Then, we compared the p- value of both models. As a result, the p-value of the second model was insignificant ($p > 0.05$) and removing those insignificant variables didn't bring any change.

Finally, a stepwise model-building strategy using the likelihood ratio test was employed, taking $p < 0.05$ for the retention of significant variables in the final model. A scatter plot was selected to check the linearity of continuous independent variables with a continuous dependent variable, i.e., calcium level. The p-value, coefficient, and standard error were used to present the findings. The results were considered significant at $p < 0.05$.

4.6. Ethical Clearance

Ethical certificate was obtained on Ethical issues regarding animal handling, sample collection and preservation from animal research ethical review committee of Addis Ababa University, College of Veterinary Medicine and Agriculture (Appendix 8).

5. RESULTS

5.1. Description of the Participants

Based on the questionnaire study, general characteristics of dairy owners are depicted in Table 3 below. From the total interviewed respondents (N = 43), the majority (65.1%) of the respondents were male, while the remaining (34.9%) were female. The majority age of the respondents in the study area ranges between 30–45 years (62.8%). Of the total dairy participants interviewed, 67.4% are married. Concerning the level of education, the highest percentage (42%) of the respondents had primary education and 6% of those respondents had basic education. 70% of the respondents had 4-6 years of experience in dairy farming.

Table 3: Background information of sampled Participants

Category and variables	Small N (%)	Medium N (%)	Large N (%)	Total N (%)
Sex				
Female	7(33.3%)	6(40%)	2(28.6%)	15(34.9%)
Male	14(66.7%)	9(60%)	5(71.4%)	28(65.1%)
Age				
< 30 Y	5(23.8%)	-	-	5(11.6%)
30-45 Y	12(57.1%)	11(73.3%)	4(57.14%)	27(62.8%)
> 45 Y	4(19.1%)	4(26.7%)	3(42.86%)	11(25.6%)
Marital status				
Single	6(28.6%)	3(20%)	-	9(20.9%)
Married	13(61.9%)	10(66.7%)	6(85.7%)	29(67.4%)
Widowed	2(9.5%)	2(13.3%)	1(14.3%)	5(11.7%)
Educational level				
Basic education	5(23.8%)	1(6.7%)	-	6(14%)
Primary education	11(52.4%)	6(40%)	1(14.3%)	18(42%)
Secondary education	3(14.3%)	5(33.3%)	2(28.6%)	10(23%)
Diploma and above	2(9.5%)	3(20%)	4(57.1%)	9(21%)

Experience in Dairy work				
1 -3 Y	5(23.8%)	2(13.3%)	-	7(16%)
4- 6 Y	13(61.9%)	12(80%)	5(71.4%)	30(70%)
> 7 Y	3(14.3%)	1(6.7%)	2(28.6%)	6(14%)

Y=Year, **N**= Number of Participants

5.2. Description of the Dairy Farms in the Study Area

Out of the 43 farms considered in this study, 48.8% (21/43) were Small-scale dairy farms, 34.9% (15/43) were Medium-scale dairy farms, and 16.3% (7/43) were Large-scale dairy farms. The number of animals in each farm size category is given in Figure (11). Regardless of the production system or farm size, almost the entire respondent agreed that feeding practice depends on the availability of feed because land space is important factors for cultivation of animal feed is limited in the farm area. Animals in all farm groups were fed natural pasture (cut-and-carry), grass hay, straw, milling byproducts (frushka), dairy concentrate mix, crop residues, and some green grasses like alfalfa and elephant grass. Also, 25% (44/176) of cows were managed semi-intensively and 75% (132/176) of cows were managed intensively in the study area.

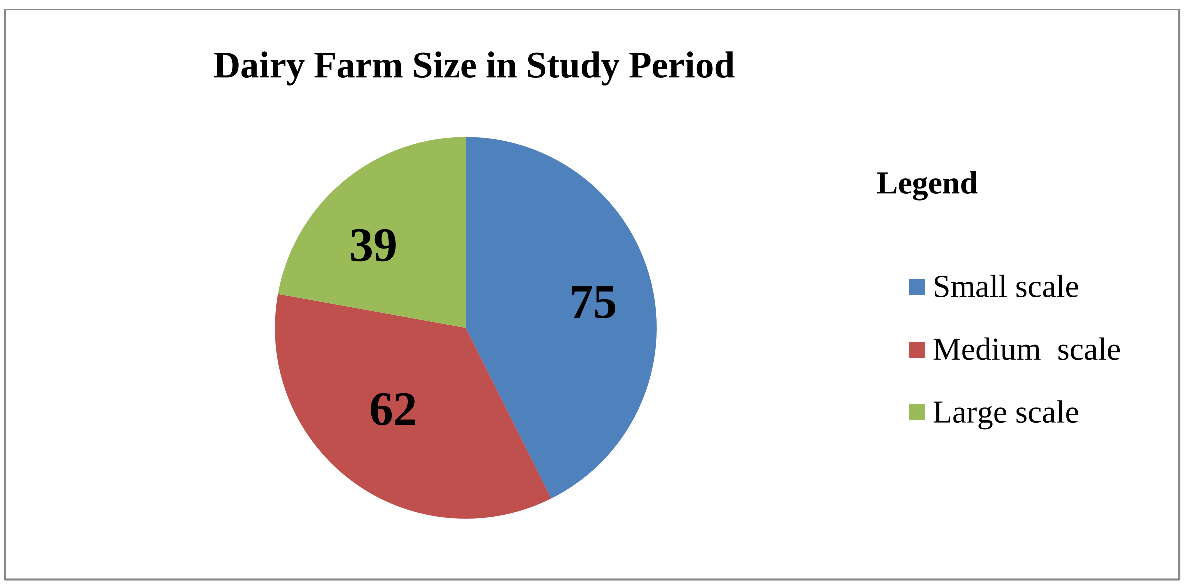


Figure 11: Number of dairy cows in dairy farm scale in the study area

5.3. Challenges/ Problems in Dairy Cow in Study Area

Almost all respondents involved in the study were asked to prioritize the challenges of dairy practice. Among the challenges which were raised by almost all respondents were the unaccessibility of vaccination, unavailability of calcium borogluconate and its cost, scarcity of supplements like calcium, feed shortage, high feed cost, and farm size, and some owners didn't bring their animals to the clinic or consult the veterinarian when their animal was sick. They opted to treat their animals by themselves, especially on small-holder dairy farms. Among the reproductive diseases of dairy that were encountered on the farm were mastitis, retained placenta, ketosis, and milk fever, which most frequently occurred.

5.4. Factors Associated with Subclinical Hypocalcaemia

Hypothesized factors related to feeding, host, and management were examined for possible association with the occurrence and distribution of subclinical hypocalcaemia in dairy cows (Table 4). As a result, some dairy cows developed SCH, which appeared clinically silent and was only diagnosed through serum analysis. Out of 176 dairy cows examined, 27.8% were affected by subclinical hypocalcaemia (49/176). Among the considered factors, breed, age, milk yield, feed type, cow status, and parity of cow were significantly associated with subclinical hypocalcaemia (Table 4).

Table 4: Descriptive statistics and Chi square test value during study

Variables	No of samples	No of SCH samples (<2.15mmol/L)	Prevalence (%)	95% CI	χ2	p-value
Feed Type						
Mixed	42	7	16.7	6-29	16.904	0.0002
Roughage	71	15	21.1	10- 29		
Concentrate	63	27	42.9	33- 58		
History of MF						
Yes	45	16	35.6	21- 50	1.3123	0.252
No	131	33	25.2	18- 33		
Herd Size						
Medium	62	16	25.8	15- 40	0.51978	0.7711
Small	75	23	30.7	20- 41		
Large	39	10	25.6	11- 37		
Age						
5-7 Year	85	16	18.9	11- 27	44.489	0.000
> 7 Year	91	33	36.3	27- 46		
Milk Yield						
5-20 Liter	95	17	17.9	10 - 27	19.42	0.0314
>20 Liter	81	32	39.5	29 - 50		
Parity						
1 and 2	33	5	15.2	3 - 27		
3 and 4	84	16	19.1	11- 27	18.551	0.0023
>4	49	28	57.1	43 - 71		
History of RFM						
Yes	57	18	31.6	19 - 44	0.34344	0.5578
No	119	31	26.1	18 - 34		
Breed						
Local	48	7	14.6	4 - 26	7.8216	0.005
Cross	128	31	24.2	17 - 32		

Body condition						
Medium	67	14	20.9	11 - 31		
Poor	26	5	19.2	3 - 35	5.4169	0.066
Good	83	30	36.1	26 - 47		
Mineral Suppl.						
Yes	44	9	20.5	8 - 33	1.1407	0.2855
No	132	40	30.3	22 - 38		
Management						
Intensive	132	38	28.8	21- 37	0.0848	0.7708
Semi-intensive	44	11	25	12 - 38		
Cow Status						
Pregnant	61	9	14.8	6 - 24		
Lactating	115	40	34.8	26 – 44	6.9929	0.008
Total	**176**	**49**	**27.8%**	**22- 35**		

As indicated in Table 4 above, the prevalence of subclinical hypocalcaemia in dairy cows those consuming mixed feed is 16.7%, roughages 21.1%, and concentrates 42.9%. Therefore, this indicates that the prevalence of subclinical hypocalcaemia is higher in dairy cows that feed concentrates than in cows that feed roughage or mixed feed types. As a result, feed type had a significant association with subclinical hypocalcaemia in the study (p = 0.0002).

The prevalence of subclinical hypocalcaemia in dairy cows with a history of milk fever is higher than in cows without a history of milk fever. However, the history of MF had no significant association with subclinical hypocalcaemia in the study (Table 4). The prevalence of subclinical hypocalcaemia in dairy cows varies with herd size (Small 30.7%, Medium 25.8%, and Large 25.6%). Therefore, this indicates that the prevalence of subclinical hypocalcaemia is higher in dairy cows with a small herd size than in cows with medium and large herd sizes. But, herd size had no significant association with subclinical hypocalcaemia in the study period (p = 0.252) Table 4.

In table 4 above, the prevalence of subclinical hypocalcaemia at age greater than 7 years was (36.3%) and age from 5 up to 7 years was 18.9%. Therefore, the age of the sampled dairy cow had a significant association with subclinical hypocalcaemia in this research (p=0.000). The prevalence of subclinical hypocalcaemia in cross-breds was 24.2%, and local was 14.6%. The prevalence of SCH in local breeds is lower than that of cross-breds. Therefore, the breed of cows had a significant association with subclinical hypocalcaemia in this research (p = 0.005).

The prevalence of subclinical hypocalcaemia in medium body condition was 20.9%, poor body condition was 19.2%, and good body condition was 36.1%. Therefore, these indicate that the prevalence of subclinical hypocalcaemia is higher in dairy cows with good body condition than in cows with poor and medium body condition. However, body condition had no significant association with subclinical hypocalcaemia in this research (p = 0.06). The prevalence of subclinical hypocalcaemia in dairy cows with parity 1 and 2 was smaller than that of parity 3 and 4, and above parity 4. Therefore, parity had a significant association with subclinical hypocalcaemia in this study period (Table 4).

As indicated in Table 4, the prevalence of SCH in cows that get mineral supplementation was 20.5% and in cows that didn’t get mineral supplementation was 30.3%. This indicates that the prevalence of SCH in cows that get mineral supplementation is lower than in those that don’t get mineral supplementation. However, mineral supplementation had no significant association with subclinical hypocalcaemia in the study (p = 0.2855).

The prevalence of subclinical hypocalcaemia in dairy cows in semi-intensive management was 25%, and in intensive management it was 28.8%. No matter how, management had no significant association with SCH in the study period (Table 4). The prevalence of subclinical hypocalcaemia in pregnant cows was 14.8% and lactating cows was 34.8%. The prevalence of SCH in lactating cows is higher than that of pregnant dairy cows. As a result, cow status had a significant association with subclinical hypocalcaemia in the study period (p = 0.008) Table 4.

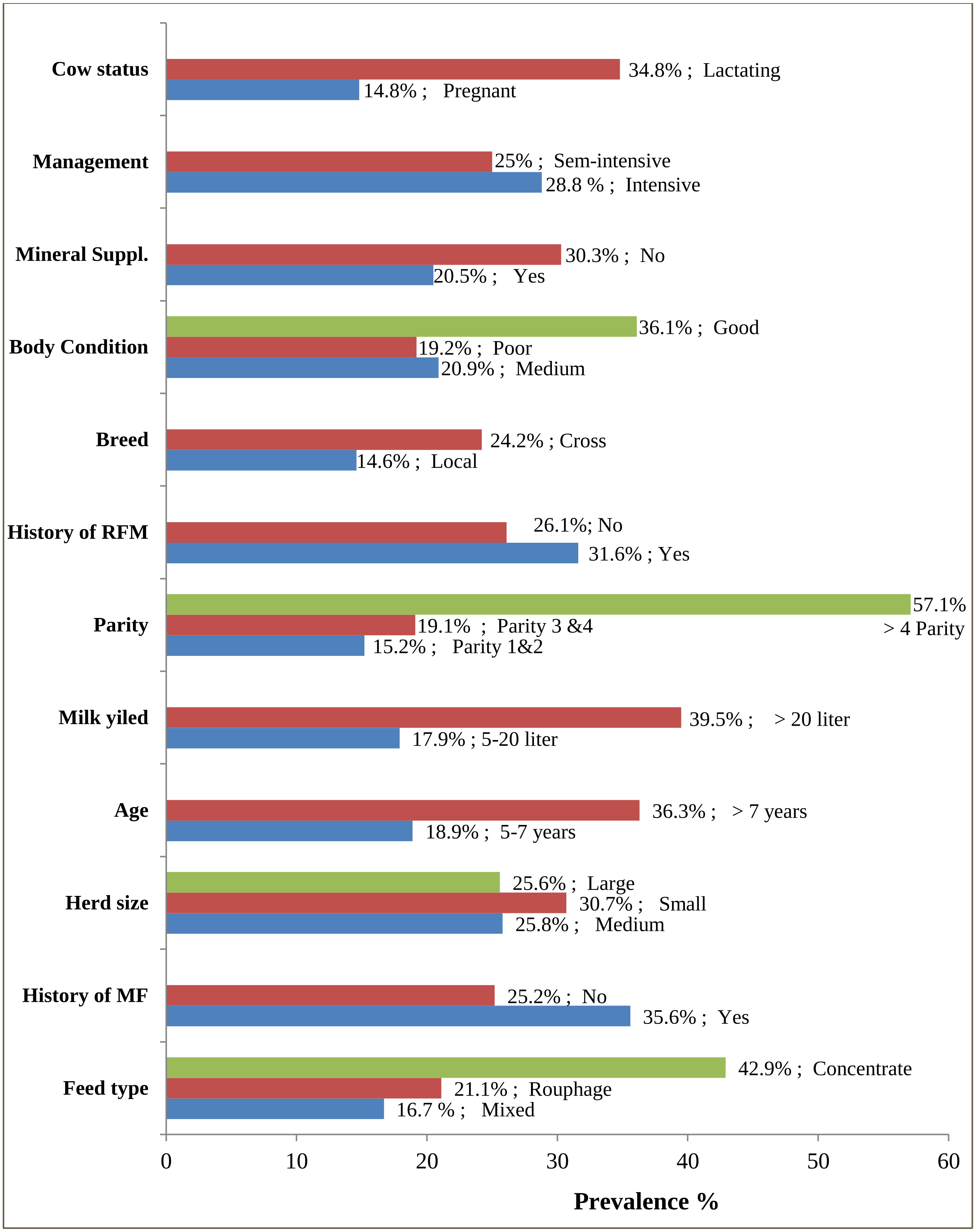


Figure12: Prevalence of risk factors observed in dairy cows affected with subclinical hypocalcaemia

5.5. Serum Calcium Level Analysis

The mean serum calcium level of the 176 cows was 2.174 mmol/L, the median was 2.21 mmol/L, the standard deviation was 0.221 mmol/L, (CI, 95% = 1.74084-2.60716), the minimum value was 1.21 mmol/L, and the maximum value was 2.49 mmol/L. Among the variables that have a significant association with serum calcium concentration are breed (local, coef =0.098740 and p=0.001), age (coef =-0.042506, p=0.000137), milk yield (coef =-0.054521, p=0.042397), feed type (mixed, coef = 0.116441 and p=0.002, roughage, coef = 0.091068 and p=0.0049), parity (coef =-0.070485 and p=0.016) and cow status (pregnant, coef = 0.055772 and p=0.0469) (Table 6).

Table 5: Simple linear regression of serum calcium concentration of dairy cow and associated risk factors (N=176)

Variables	Coefficient	Std.Error	t -value	p –value
Feed type				
Mixed	0.23892	0.03946	6.055	0.00005
Roughages	0.20285	0.03340	6.073	0.00048
Concentrates		**Ref.**		
History of MF				
Yes	-0.06760	0.03796	-1.781	0.0767
No		**Ref.**		
Herd size				
Medium	-0.02297	0.04525	-0.508	0.612
Small	-0.05090	0.04371	-1.164	0.246
Large		**Ref.**		
Age	-0.057489	0.00643	-8.931	0.000
Breed				
Local	0.20488	0.03472	5.901	0.0002
Cross		**Ref.**		

Body condition				
Medium	0.06126	0.03622	1.691	0.0926
Poor	0.03042	0.04956	0.614	0.5402
Good		**Ref.**		
Parity	-0.060485	0.008705	-6.948	0.000
Milk yield	-0.01134	0.002491	-4.555	0.000
History of RFM				
Yes	-0.005349	0.035711	-0.15	0.881
No		**Ref.**		
Mineral suppl.				
Yes	0.04515	0.03844	1.175	0.242
No		**Ref.**		
Management				
Semi-intensive	0.04364	0.03845	1.135	0.258
Intensive		**Ref.**		
Cow Status				
Pregnant	0.12862	0.03374	3.812	0.00019
Lactating		**Ref.**		

N= number of dairy cow **Ref. =** Reference

The Table 5 above indicated to show the simple linear regression of serum calcium concentration of dairy cow and associated risk factors during study period.

Table 6: The final multivariable linear regression model evaluated the relationship between serum calcium concentration and significant variables**.**

Variables	Coefficient	Std.Error	t-value	p-value	CI, 95%	
Age	-0.042507	0.010889	-3.904	0.000137	-0.0638	-0.0211
Breed						
Local	0.098740	0.031221	3.163	0.001856	0.0375	0.1599
Cross		**Ref.**				
Milk yield	-0.054521	0.032314	-1.69	0.042397	-0.1179	0.00881
Feed type						
Mixed	0.116441	0.037095	3.139	0.002003	0.0437	0.18915
Roughage	0.091068	0.031955	2.850	0.004922	0.0284	0.1537
Concentrate		**Ref.**				
Parity	-0.070485	0.008105	-8.69	0.01632	-0.0863	-0.0546
Cow status						
Pregnant	0.055772	0.027859	2.002	0.046901	0.0011	0.1104
Lactating		**Ref.**				

Ref. = Reference

The result of Table (6) was finally explained in the discussion part of this research. To indicate clearly, the coefficient and p-values of the above result are an important point that was explained in the discussion part of this study. Because coefficients are values that measure the magnitude of a variable, whereas p-value indicates the significance/association of the predictor variable with the response variable (outcome). Therefore, the above table was the final output/model of this study period.

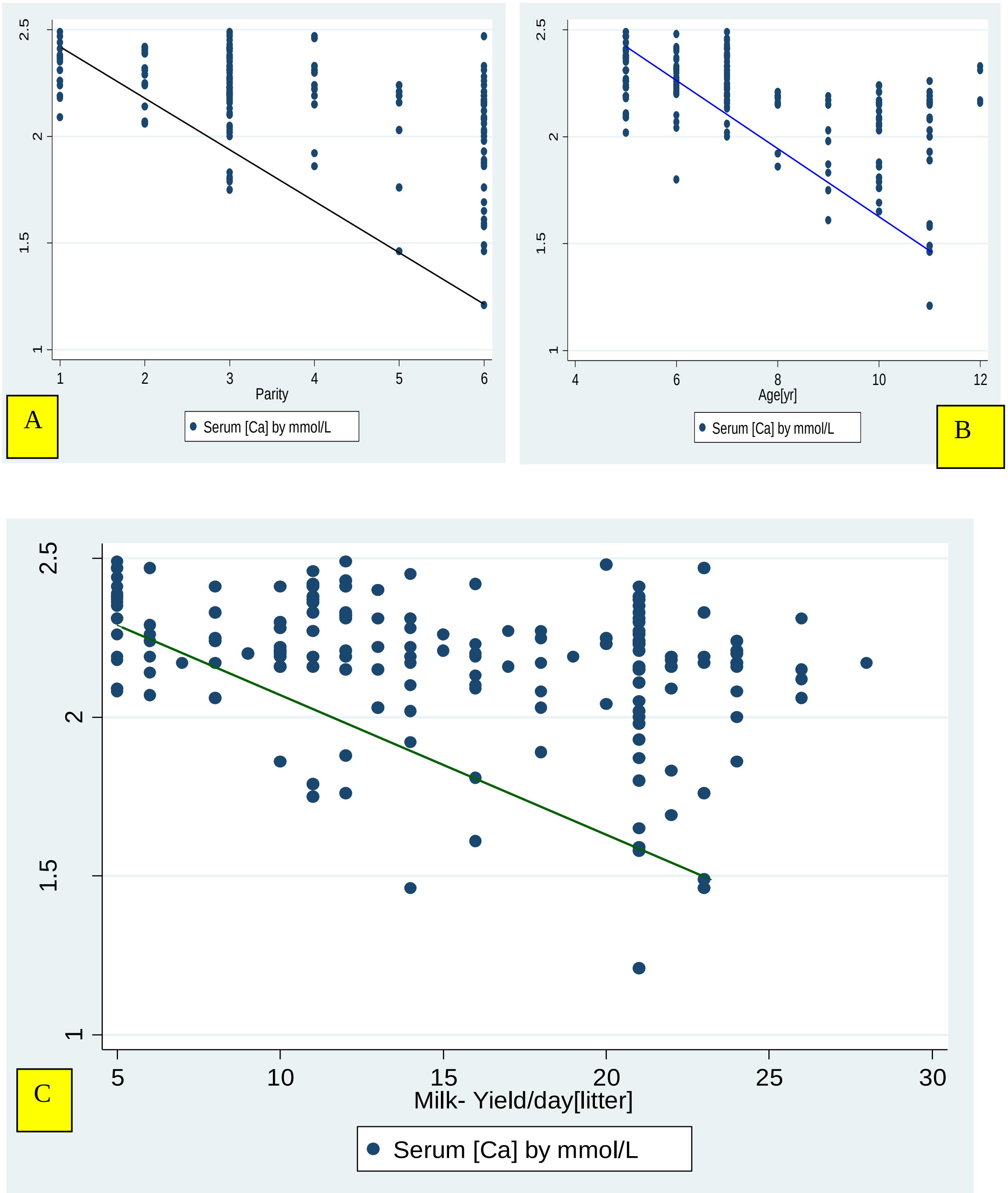
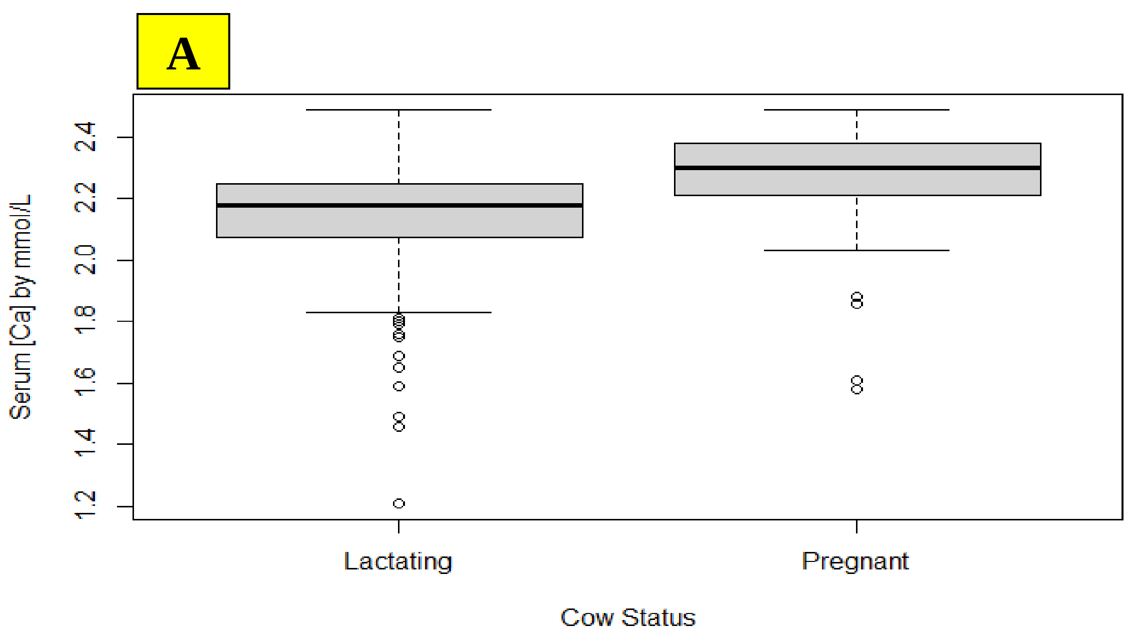


Figure 13: Scatter plot of continuous independent variables that are significant impact on variable of interest (serum calcium level).

The median for the dairy cows that are pregnant is close to the upper adjacent value for the cows that are lactating, which leads to the conclusion that the serum calcium concentration varies with cow status. The main difference between the groups lies in their different concentrations around the median rather than their overall spread of values. The potential outliers for the cow with pregnancy are not very far from the lower adjacent value for the lactating cow, and they are marked as outliers essentially because of the comparatively low interquartile range for the sample into which they fall. The overall conclusion is that serum calcium concentration varies according to cow status (Figure 14 (A)).

The median for local-bred dairy cows is higher than the median for cross-bred dairy cows. In other words, the median of local breeds is close to the upper adjacent value for crossbreeds, which leads to the conclusion that the serum calcium concentration varies with breed type. The main difference between the groups lies in their different concentrations around the median rather than their overall spread of values. The potential outliers for the local breed are not very far from the lower adjacent value for the cross breed and are marked as outliers. The overall conclusion is that the serum calcium concentration does vary with the breed type of dairy cow (Figure 14 (B)). The median serum calcium concentration of dairy cows fed concentrate is lower than those fed mixed and roughage feed types, implying that serum calcium concentration varies with feed type (Figure 14 (C)).



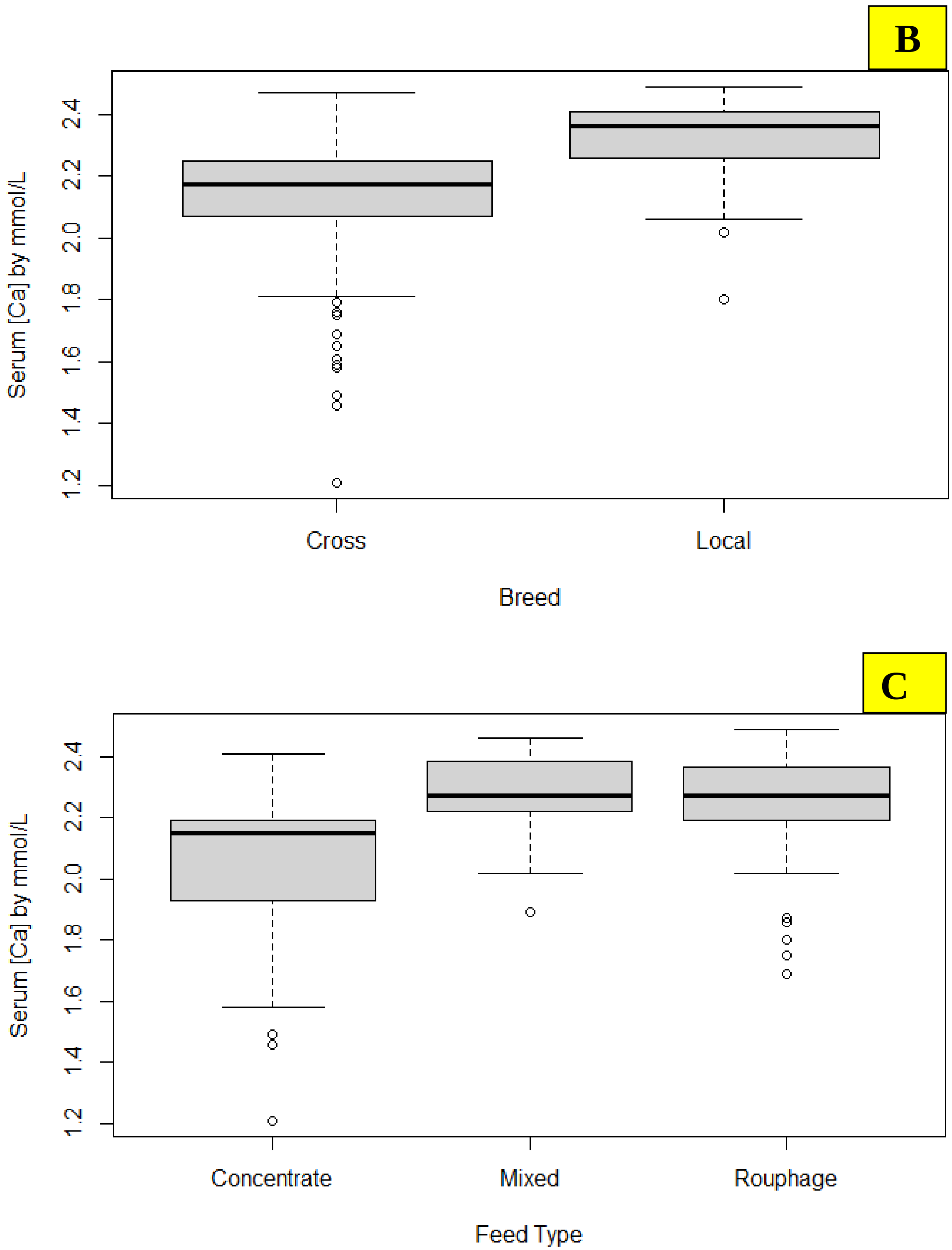


Figure 14: Box plot of categorized independent variables with continuous dependent variable that are significant impact on variable of interest (outcome).

6. DISCUSSION

The transition period continues to be a significant time period for cow health, with implications for current and future production in the dairy cattle sector all over the world. The ability to identify the risk factors for subclinical hypocalcaemia and related blood component problems in cows during the transition period allows for early intervention and prevention of the disease occurrence. So, the present study revealed that, 27.8% of cows had a serum calcium concentration less than 2.15 mmol/L or the overall prevalence of subclinical hypocalcaemia in the study was 27.8%, which in line with previous reports of 30% in Bangladesh (Naher *et al.*, 2020), 26.1% in Ethiopia (Adugna, 2019), 20.3% in Ethiopia (Mekonnen *et al.*, 2022), and 25% to 54% in the United States of America (Reinhard *et al.*, 2011). But, the result was lower than the finding of (da Silva *et al.*, 2019) which was 40.34% in Brazil and 47.6% (Venjakob *et al.*, 2017) in Germany. This variation is attributable to sampling occurring early in the calving process, notably in the first 48 hours, as well as a difference in production system and cow breed that made comparison impossible (Mekonnen *et al.*, 2022).

During the study, the breed of cow was significantly associated with subclinical hypocalcaemia ($\chi2$= 7.8216, p=0.005). The prevalence of subclinical hypocalcaemia in cross-bred cows is 24.2%, which is higher than that of local breeds at 14.6% (Table 4). Similarly, the breed of cow has a significant association with serum calcium concentration during the study period. The local breed coefficient (0.098740) indicates that the average serum calcium concentration of the local breed was 0.098740mmol/L higher than the cross-bred (Table 6). These results are similar to those of Akalu (2017). This variation is associated with higher milk production per unit of body weight and a reduction of intestinal vitamin D_3 receptors in cross-bred cows as compared with local breeds (Peterson and Beede, 2002).

This study was demonstrated that there was no association between subclinical hypocalcaemia and the body condition of dairy cows ($\chi2$= 5.4169, p=0.06). This finding contradicts the findings of (Ostergaard *et al.*, 2003; Naher *et al.*, 2020; Abdelmenem *et al.*, 2020), who included more samples in good body condition than the other groups. But, by chance, the body condition of sampled dairy was almost near each other, which made this factor insignificant in this finding. However, this finding is similar to the previous report of (Mekonnen *et al.*, 2022).

The cow status (stage of transition period) of the sampled dairy cow had a significant association with subclinical hypocalcaemia, as from the calving day till 3 weeks post-partum was the significant time zone for its occurrence (34.8%) compared to the period of 3 weeks prior to the EDD, which was 14.8% ($\chi 2=6.9929$, $p=0.008$) (Table 4). By using linear regression, cow status had a significant association with serum calcium concentration. The coefficient of a pregnant cow in this study period indicated that the average serum calcium concentration is increased by 0.0568457mmol/L as compared to an early lactating cow (Table 6). This research is in line with the previous report by Abdelmenem *et al.*, 2020. This may be due to the daily milk yield significantly affecting the incidence of hypocalcaemia in dairy cows under investigation.

There was a statistically significant relationship between the age of the sampled dairy cow and subclinical hypocalcaemia ($\chi 2=44.489$, $p=0.000$). The prevalence of subclinical hypocalcaemia above 7 years of age was (36.3%) and from 5 up to 7 years of age was 18.9%. These indicate that age has an influence on the occurrence of subclinical hypocalcaemia (Table 4). From the linear regression model, the age of the sampled dairy cow has a significant association with serum calcium concentration. The coefficient of age indicated that as a sampled dairy cow's age increased by one year, its average serum calcium concentrations was decreased by 0.042507mmol/L (Table 6 and Figure 13 (B)). These results agree with the previous report (Nahe r *et al*., 2020), which indicated the highest prevalence of subclinical hypocalcaemia in older age. This may be associated with vitamin D and PTH, which are the chief players in the calcium homeostasis mechanism. A decrease in the number of vitamin D receptors in the intestine has been reported with increasing age of the cows as well as the PTH receptors in the target tissues (Horst *et al.*, 1990).

In the study period, parity had a significant association with subclinical hypocalcaemia ($\chi 2=18.551$, $p=0.0023$). The prevalence of subclinical hypocalcaemia in parities 1 and 2 was 15.2%, in parities 3 and 4 it was 19.1%, and above parity 4 it was 57.1%. These indicate that parity has an influence on the occurrence of subclinical hypocalcaemia in the study (Table 4). Consequently, parity has a significant association with the serum calcium concentration of cows. The parity coefficient (-0.060485) indicates that as a sampled dairy cow's parity increased by one unit, the average serum calcium concentration decreased by 0.060485mmol/L while all other variables remained constant (Table 6 and Figure 13 (A)). These reports are in line with the previous reports (Curtis *et al.,* 1985; Reinhardt *et al.,* 2011). There was another report which supported the present finding that, as the lactation period of dairy cows increased, the serum calcium concentration decreased (Neves *et al.,* 2017; Venjakob *et al.,* 2019; Naher *et al.,* 2020). Those reports indicated that cows on high parity were more likely to suffer from sub clinical hypocalcaemia than cows on low parity. This may be associated with an increase in parity, prone to high milk production, which in turn depletes calcium through milk.

The milk yield of sampled dairy cows had a significant association with subclinical hypocalcaemia cows during investigations ($\chi2$=19.42, p = 0.0314). The prevalence of subclinical hypocalcaemia in dairy cows of 5–20 liter milk yield and above 20 liter milk yield was 17.9% and 39.5%, respectively. This suggests that the milk yield of a dairy cow sampled can influence the occurrence of subclinical hypocalcaemia (Table 4). By using linear regression, the milk yield of sampled dairy was significantly associated with serum calcium concentration during the study. The milk yield coefficient (-0.054521) indicates that as the milk yield of a sampled dairy cow increased by one liter, the average serum calcium concentration of sampled dairy cow was decreased by 0.054521 mmol/L, while all other variables remained constant (Table 6 and Figure 13(C)). This implies that milk yield was one of the factors that dropped the serum calcium level for the occurrence of subclinical hypocalcaemia in the study. This study was in line with the previous reports (Jawor *et al.*, 2012; Adugna, 2019; McArt and Neves, 2020).

The feed type of a dairy cow had a significant association with subclinical hypocalcaemia during the investigation ($\chi2$= 16.904, p=0.0002). The prevalence of subclinical hypocalcaemia in dairy cows consuming mixed, roughage, and concentrate feed was 16.7%, 21.1%, and 42.9%, respectively. This implies that, the feed type of dairy cow can influence the occurrence of subclinical hypocalcaemia (Table 4). In the linear regression model, the feed type of dairy cow was significantly associated with serum calcium concentration during the study time. The coefficients of cows that consumed mixed and roughage types of feed indicate that the average serum calcium concentration of sampled dairy cows increased by 0.116441mmol/L and 0.091068 mmol/L, respectively, as compared to cows that fed concentrates (Table 6). This study was in line with the previous report (Ribeiro *et al.*, 2013), which indicated that dairy cows whose feed concentrate are more prone to milk fever than others. This may be related to some roughage types of feed having higher calcium concentrations than other dairy cow feeds. A report by

Abdelmenem *et al.* (2020) also indicates that concentrated feeds had a greater impact on the occurrence of hypocalcaemia during the transition period.

7. CONCLUSION AND RECCOMMENDATIONS

The present study revealed that subclinical hypocalcemia is a prevalent metabolic condition affecting 27.8% (n = 49) of dairy cows in Bishoftu area. Among hypothesized factors, breed, age, parity, feed type, cow status, and milk yield of dairy cows were significantly associated with the prevalence of subclinical hypocalcaemia. Accordingly, the condition was significantly higher number cases were detected in dairy cows that were in transition period, aged above 7 years, cross breed and/or cows with four and above parity numbers. The study also found that as the age, parity, and milk yield of dairy cows increased, their mean serum calcium level becomes decreased. Dairy cows examined in present study did not show any clinical signs of hypocalcaemia and as a result farmers did not prioritize subclinical hypocalcaemia as major challenge of the dairy cows. Literatures confirm subclinical hypocalcemia as one of the gateway diseases and predispose the cows to various metabolic and infectious disorders during the transition period. Therefore, based on the above conclusion the following recommendations were suggested:

- Government sectors should give attention to milk fever and should make the drugs (e.g. calcium borogluconate, calcium oral gel etc.) available in the market at reasonable costs.
- Future research should be on another electrolyte like magnesium concentration that is associated with milk fever.
- A metabolite profile test should be performed on a regular basis during the transition period of a high-yield producing dairy cow.
- Emphasis should be given on the nutritional management of dairy cows in the dry period as well as at parturition.
- Attention should be given to those factors that have a significant impact on the occurrence of disease.
- Instrumentation should be available in the sector (especially in universities) for serum electrolyte analysis for the better advantage of dairy owners and producers.

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9. APPENDICES

Appendix 1: Blood sample collection format for individual cow

Region__________Zone__________District_________species______PA__________Date ______________

Code	Herd size	Age	Feed source	Breed	BCS	Parity	Management	Milkyield /day	Serum **Ca** concentration

BCS = Body condition score Ca= Calcium

Appendix 2: Questionnaires for individual cow and owners.

A. Background Information of Owners

Region_________ Zone____________ District ______________ species_____

Village/ PA ____________

Respondent Name________ Age ______ Sex_____ Marital status__________

Responsibility in the house holds _______ Occupation ______ Education level _____

Owner experience (years) _____

Family size_____

B. Information about cow.

1. Breed of livestock kept. A) Local, B) Cross

2. What is the source of feed for cow?

A) Hay B) Straw C) Concentrate D) Concentrate and hay or straw (mixed)

3. Herd composition or Herd inventory _____

→cow status: dry (non-pregnant) _____ pregnant_____ Lactating_____

4. What is the frequency of cow milking?

A) Ones a day B) twice a day C) three times a day

5. Did any of your cows suffer from milk fever?

A) Yes B) No,

If yes, answer the following question:

What day of the cow's milk fever occurred?

A) Before 3 day B) 3 to 6 day C) over 6 days

What is level of milk production before suffered from milk fever?

A) 10 liter per-day B) 10-20liter per day C) >20 liter per-day

What is level of milk production after suffered from milk fever?

A) 10 liter per-day B) 10-20liter per- day C) >20 liter per-day

6. What is the level of cow milk per day?

A) 5-10 liter B) 10-20liter C) >20 liter

7. What is the number of cows calving?

A) 1 B) 2 C) 3 D) 4 E) 5 F) 6 G) 7

8. What is management system of your cow?

A) Intensive B) semi-intensive C) extensive

9. Water points in different seasons:

→Dry Seasons: A) River B) Ponds C) Traditional wells D) Others: Frequencies (days) ___

→Wet Seasons: A) River B) Ponds C) Traditional wells D) Others: Frequencies (days) ____

10. What is the main means of health care for your cow?

A) Traditional healer B) self-administered vet drugs C) Veterinary clinic

11. Have you ever seen metabolic diseases and or disorders in your animal?

A) Yes B) No →If yes, what are they (list them) ________________________________

→What are the signs that indicate such diseases? _____________________________________

12. Did you have a blood test of your cow during pregnancy? A? Yes B) No

13. Do you encounter metabolic disease events (Abortion, ketosis, milk fever, dystocia, bloat and Retained placenta) in your herd?

→Since one years A) Yes B) No C) Number_______

→Since three years A) Yes B) No C) Number_______

14. What do you think that cause of recumbence in cow?

____

What do you do with cow recumbent? A) Consult veterinarians B) slaughter

C) Keeping D) Other _______

15. How do you herd your Cow? A) Separately B) with village herd C) with cow in the farm

16. How many times was an animal feed per day? _________________

17. Did your cow die from milk fever? A) Yes b) No

If yes, at what times?

A) Before parturition period B) after parturition period C) during lactation period

18. Did your cow decrease milk production? A) Yes b) NO

If yes, what is the reason? _____________________

19. Did you have mineral supplementations for your cows? A) Yes B) NO

20. What do you think the government (agriculture, universities, etc.) and private sectors should do to support improve productivity of your cow? ________________________________

21. Do you have any comment?

____

Appendix 3: Principle, working solution, instrumentation during sample analysis

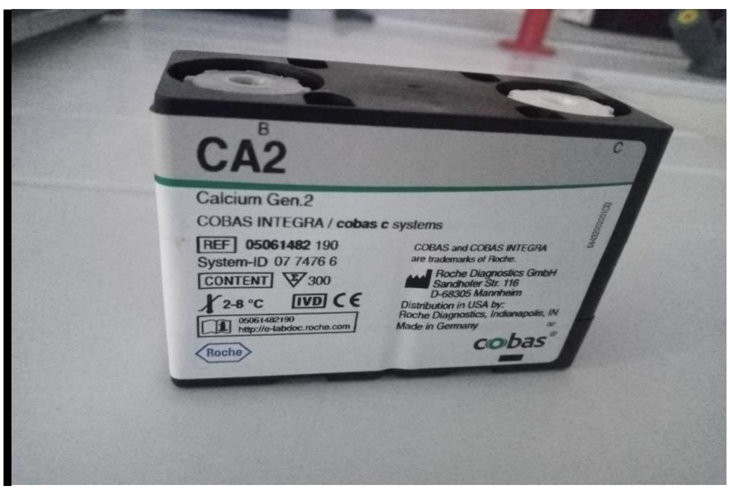
- **Test principle**

  - Calcium ions react with 5-nitro-5'-methyl-BAPTA (NM-BAPTA) under alkaline conditions to form a complex. This complex reacts in the second step with EDTA. The change in absorbance is directly proportional to the calcium concentration and is measured photometrical.

Alkaline PH

  - Ca^{2+} +NM-BAPTA calcium ⟶ -NM-BAPTA complex
  - Calcium-NM-BAPTA complex +EDTA ⟶ NM-BAPTA + calcium EDTA complex
  - The change in absorbance is directly proportional to the calcium concentration and is measured photometrical.

- **Reagents- working solutions**
  - Calcium Gen.2, 300 tests – the reagent cassette is labeled as CA2. R1 is in position B and R2 is position C



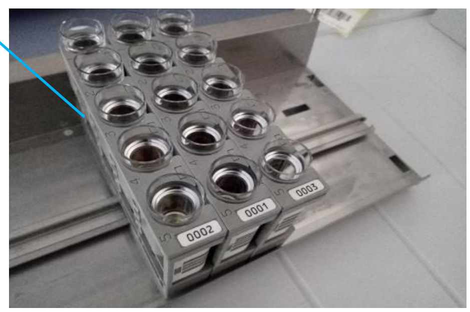
  - **R1:** CAPSO(3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid): 557 mmol/L; NM-BAPTA: 2mmol/L, pH 10.0; non-reactive surfactant and stabilizer
  - **R2:** EDTA: 7.5 mmol/L, pH 7.3; non-reactive surfactant, preservative

- Diluent NaCl 9%, 50 mL – the diluent cassette is labeled as NACL

➢ **Equipment/Instrumentation**:

- Roche c501 analyzer

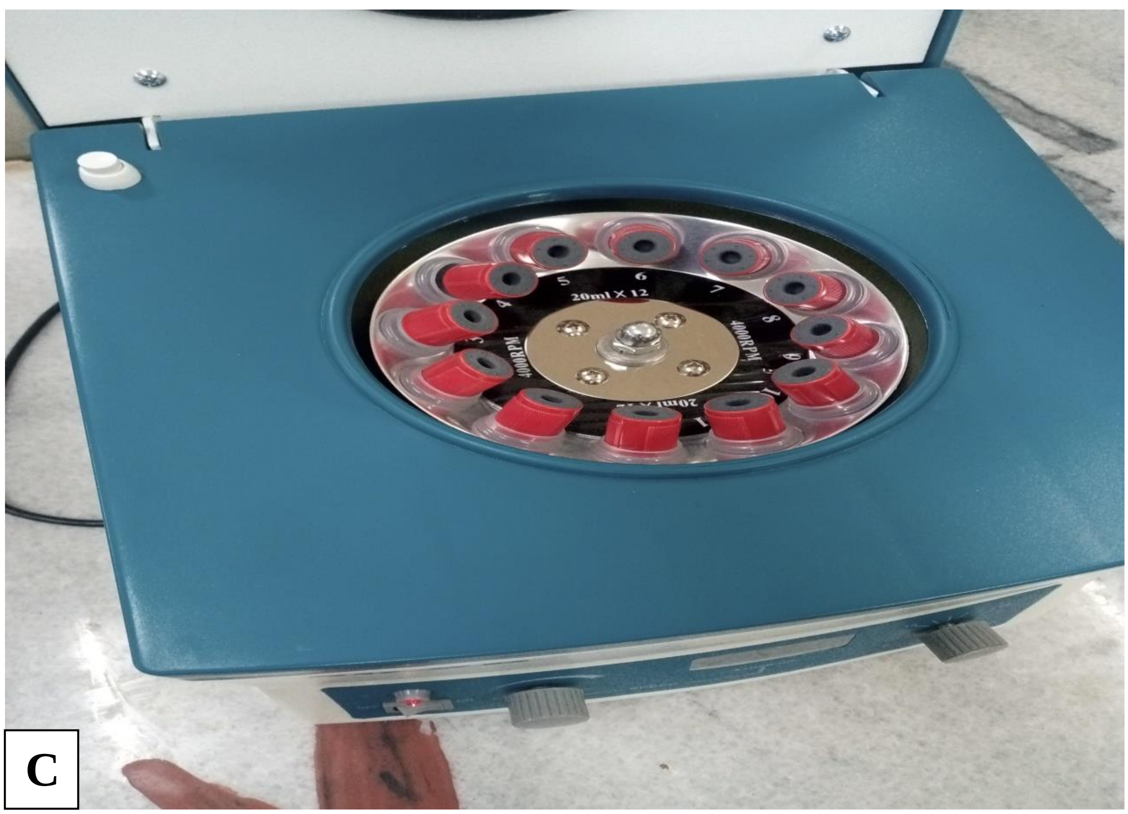
- Sample rack



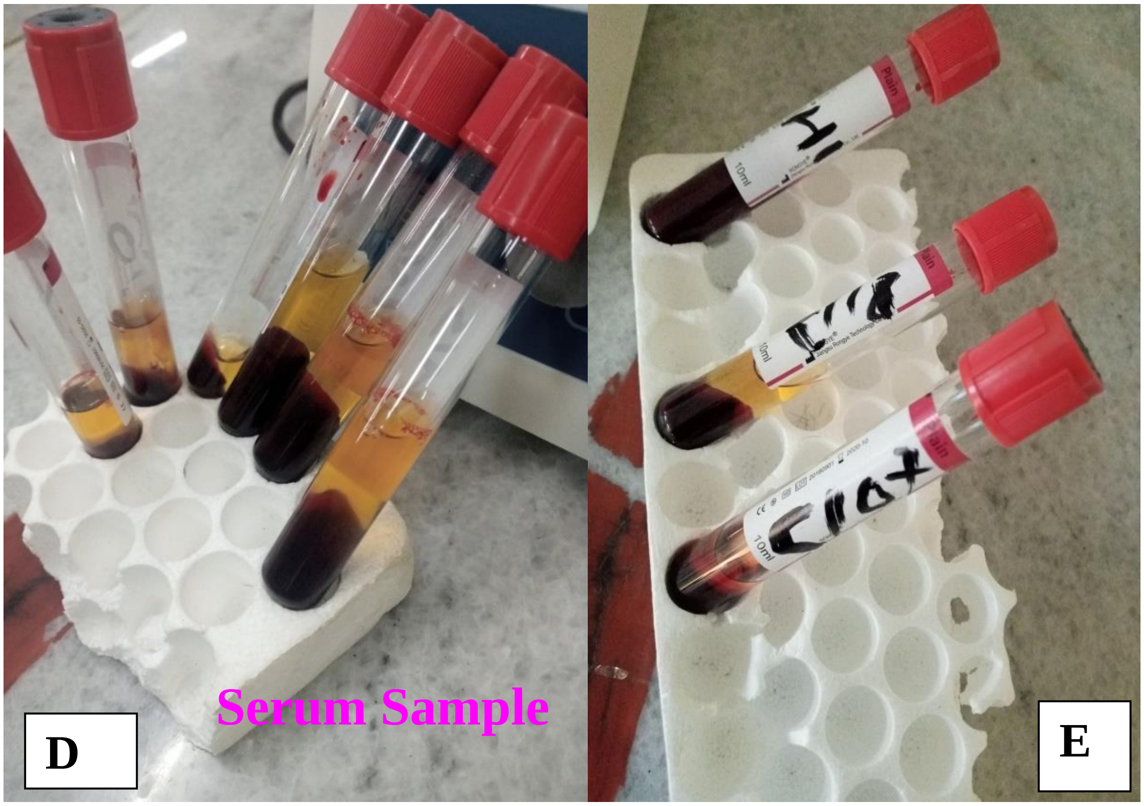
- Calibration mode: Linear, 2-point calibration.
- Calibrators: C.f.a.s. Use deionized water as zero calibrator
- Calibration Frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than twenty-four hours since the reagent pack was registered on the analyzer). Renewed calibration is recommended as follows:
  - ✓ If necessary after instrument service or repair
  - ✓ If dictated by quality control results

Appendix 4: <u>Some selected photo during sample collection and its preparation at laboratory</u>

Picture taken during blood collection from the field during study period

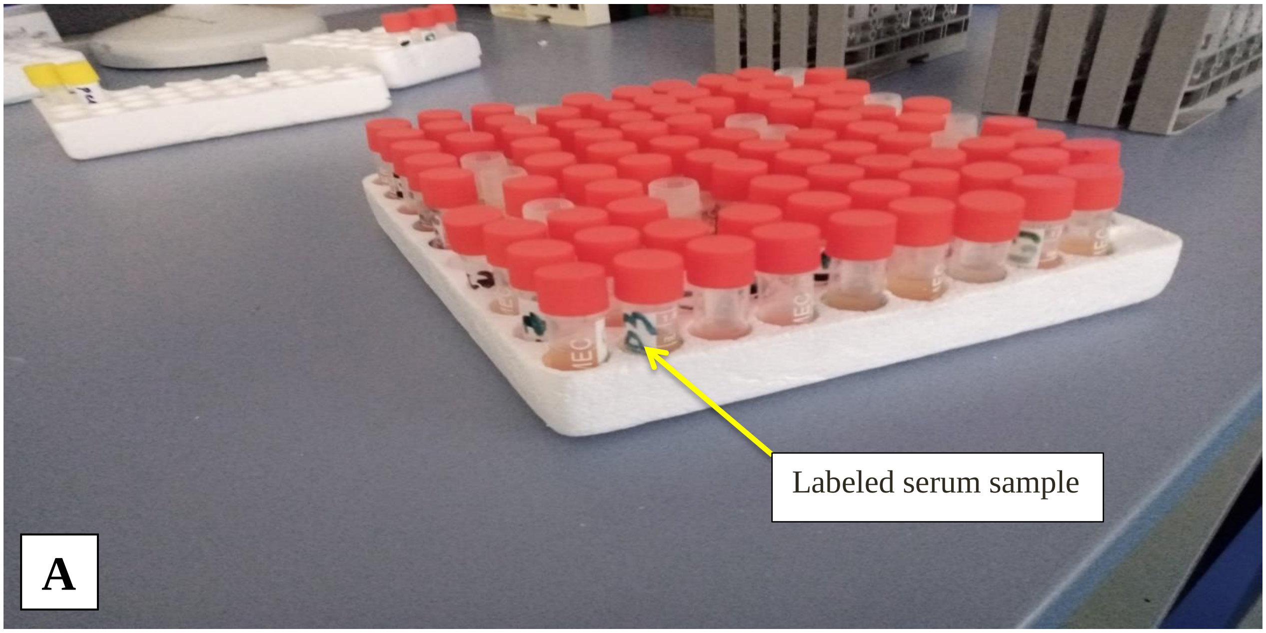


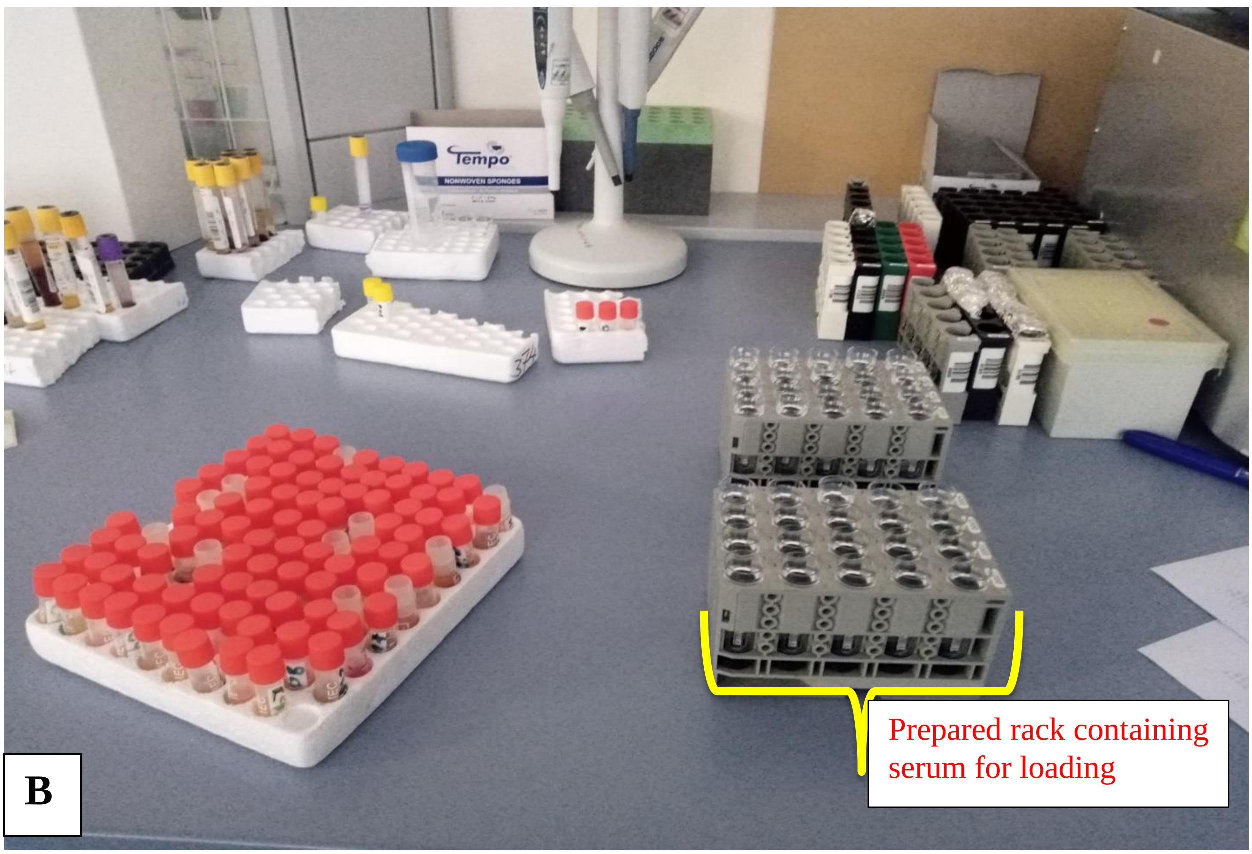
Picture taken during blood centrifuge Machine with balanced sample



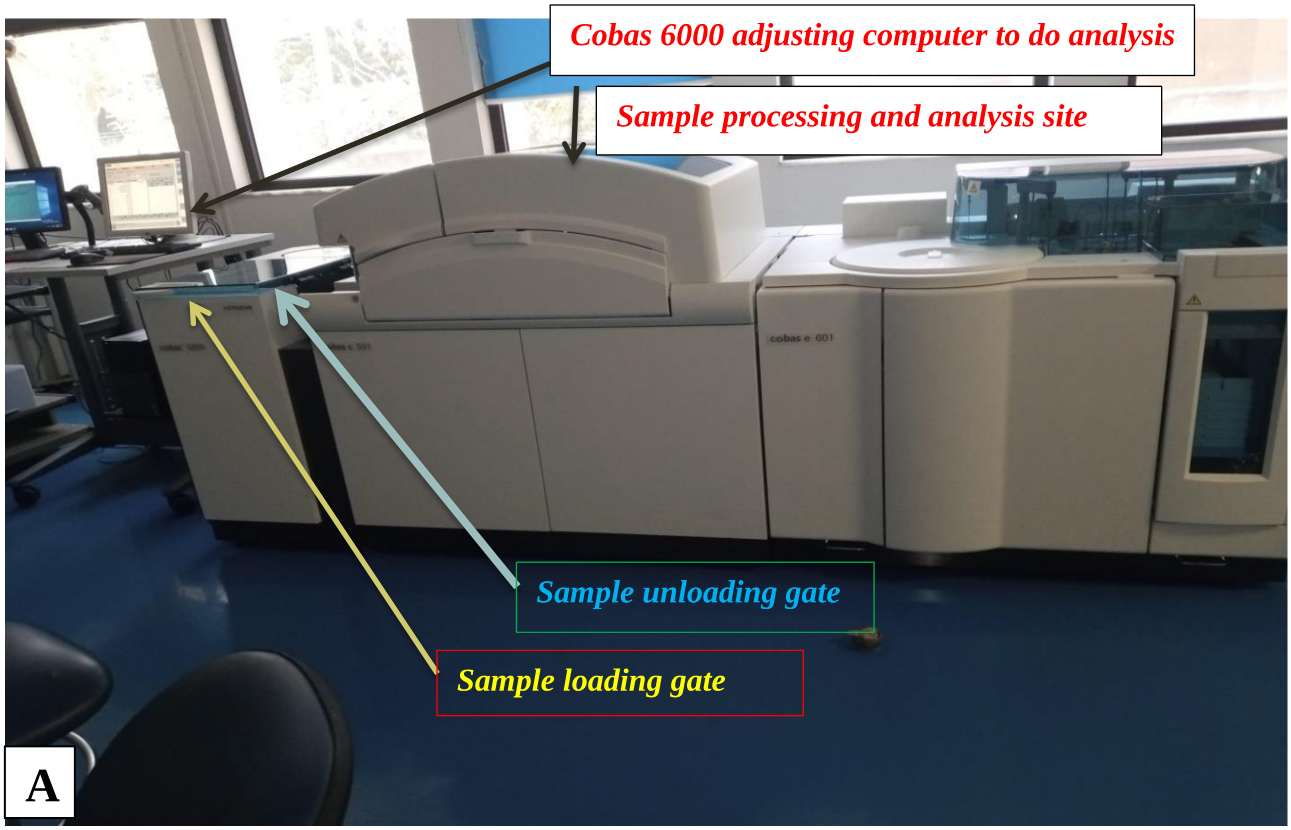
Picture of Centrifuged blood taken at laboratory during serum extraction

Appendix 5: Some selected photo while serum ready for analysis at APHRLC





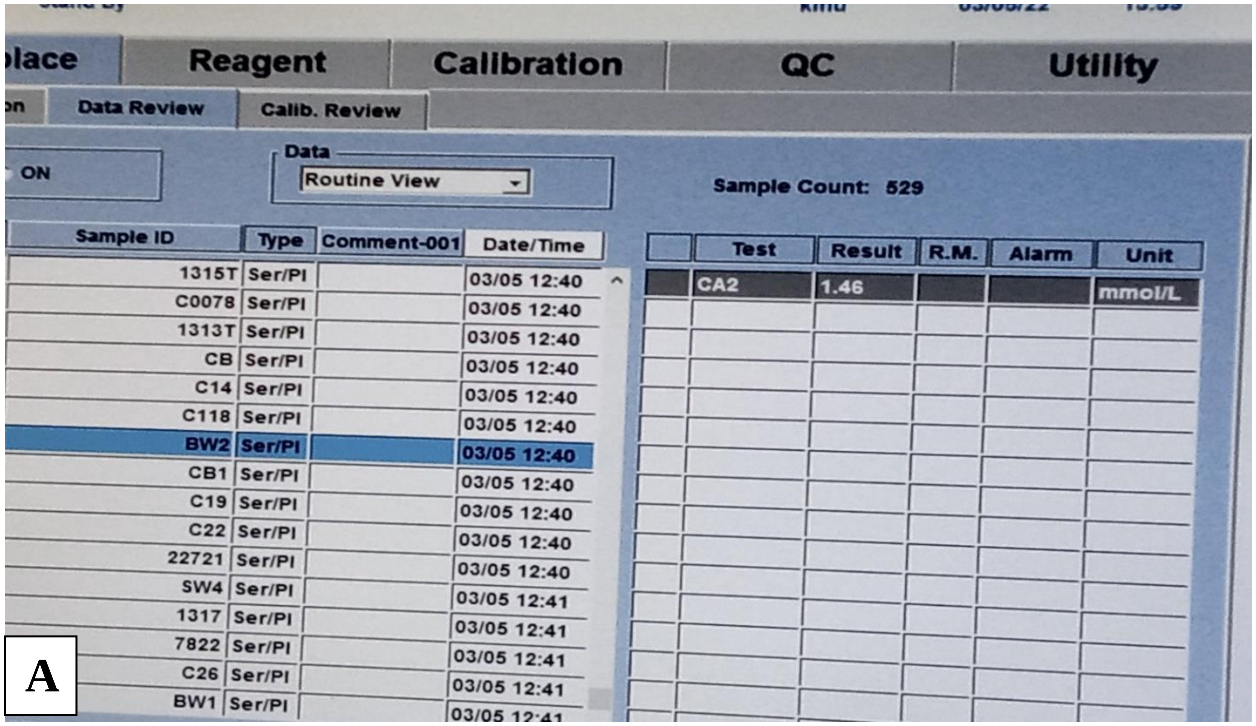
Appendix 6: Some selected photo during sample process and analysis at APHRLR



Picture of Cobas 6000 electrolyte machine taken during sample analysis at APHRLC.

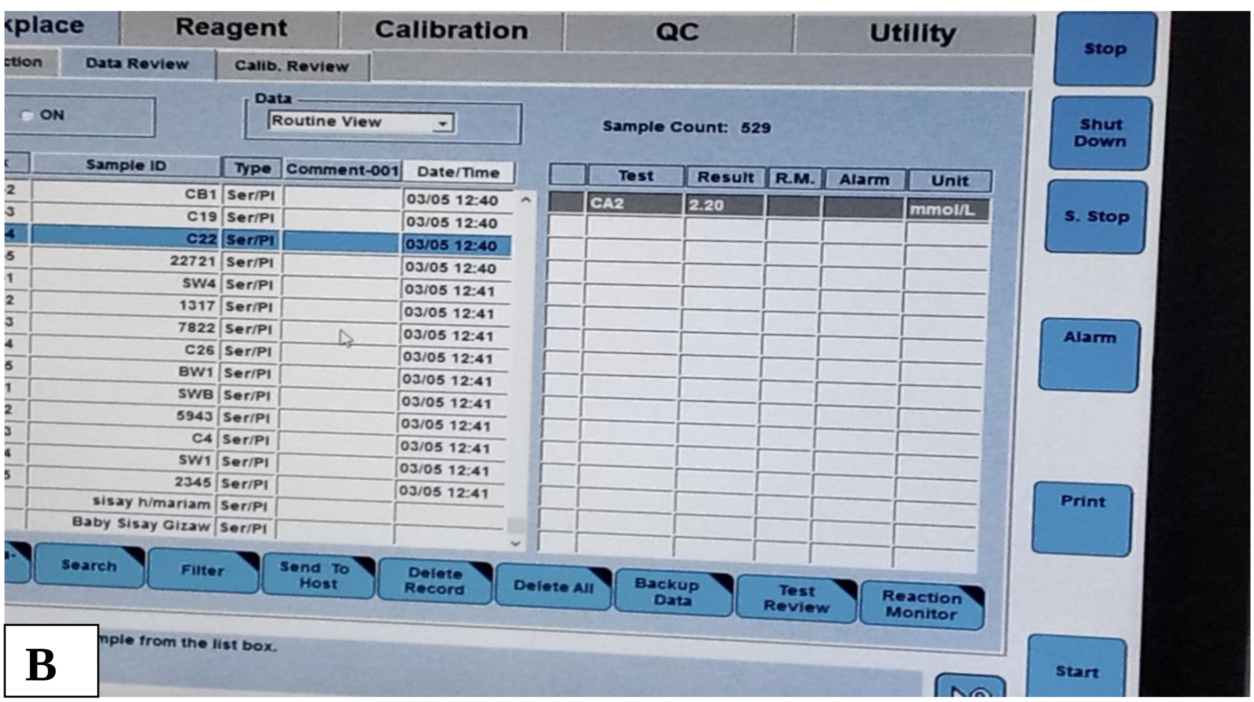
Picture taken during sample code recoding with sample rack reference number

Appendix 7: Some selected result of serum sample during analysis at APHRLC



A

Picture of sample considered as subclinical hypocalcaemia (<2.15 mmol/L) at APHRLC



B

Picture of sample considered as normocalcaemic cow (2.15 - 2.5 mmol/L) at APHRLC

Appendix 8: Ethical clearance 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/09/02/14/2022

Name of Applicant: **Mogos Mekonnin Jima (DVM, MSc fellow)**

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

Title of the project: *Epidemiology of subclinical hypocalcemia and its metabolite profile in dairy cow during transition period in and around Bishoftu town*

Date of application:	**December, 2021**
Nature of the project:	**Mildly invasive**
Target animal species:	**Dairy cows**
Number of animals involved:	**176**
Study area:	Bishoftu, **Ethiopia**

Minutes No. and date of review: **VM/ERC/02/14/022, 01/03/2022**

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, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

Professor Getachew Terefe (DVM, PhD)
Chairman

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

Signature

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ፋክስ}
Fax 251-11-4339933
ስልክ }
Tel. +251 114338450
ፖ.ሣ.ቁ
P.o.x. Box}34
ቢሾፍቱ፤ ኢትዮጵያ
Bishoftu, Ethiopia