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A Study of Relationship Of Prolactin And kappa Casein Genes Polymorphism Separately On Some Physiological And Milk Production Traits On Local Cows In Karbala Province

A THESIS

Submitted to the Council of The College of Veterinary Medicine, University of Karbala in Partial Fulfillment of the Requirements For the Degree of Master of Science In Veterinary Medicine/ Physiology

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dedication

to :

My deceased father and my dear mother...for their efforts and kindness.

They supported me and supported me in the most difficult circumstances and taught me patience until I achieve success and progress.

My dear wife.....

Who stood with me in the most difficult times. I am very grateful for your tolerance, assistance and generous support

My dear teacher and supervisor... I thank my dear brother for anything you have given me.

My brothers (Amir and Boys)...

Who were a source of constant support and encouragement to me and their presence in my life is a blessing from God.

Dear friends... Thank you all for your support to me.

Which added a block on top of what was built.

I dedicate my humble efforts to you.

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Haider

summary

Summary:

The aim of this study was conducted to investigate the role of prolactin and kappa casein genes polymorphism associated with milk traits and some hormones concentration and also investigate the effect of prolactin and kappa casein genes polymorphism on milk quality and quantity minerals concentration such as protein, lactose, freezing point, density, fat, solid non fat and Czen, and with some trace elements such as Serum Iron (Fe), Serum Phosphorus (P) and Serum Calcium (Ca), and with some hormones concentration such as Prolactin (PRL), Growth hormone (GH), Thyroid stimulating hormone (TSH), Progesterone hormone (PRO), Estrogen hormone (Es), oxytocin hormone (OXY), and also investigate the effect of prolactin and kappa casein genes polymorphism on some blood parameters namely Red blood cell count (RBC), Packed cell volume (PCV), Hemoglobin cancalated (Hb), mean corpuscular volume (MCV), mean corpuscular Hemoglobin concentration (MCHC), and White blood cell (WBC) dairy cows in Karbala province.

This study is the first of its kind in Iraq. One hundred twenty healthy dairy cows were used to collect serum and blood sample in lactation period in stage (40-120) days during December 2020 to May 2021 in the northeast of rural areas in Karbala province in Iraq.

The blood samples were classified after detecting prolactin gene and kappa casein gene polymorphism used by amplification refractory mutation system (ARMS- PCR) techniques and RFLP-PCR techniques to three genotypes groups for prolactin gene polymorphism as a wild group (AA), recessive group (AG), and heterozygosity group (GG), also kappa casein gene polymorphism to three genotypes group to wild AA, recessive AB group and heterozygosity BB group.

Our result there shows a significant decrease in the protein, lactose, fat and solid non fat percentage and density in AG, GG, AB and BB compared with Wild genotype group.

On the other hand the current result also revealed a significant increase ($p \leq 0.05$) in serum concentration in Iron with AA group compared with AG,GG, AB, BB while in serum phosphorus there was an increase in AA groups of kappa casein gene polymorphism compared with AB, BB groups and in serum concentration of calcium was no significant in prolactin and kappa casein gene polymorphism.

Our results showed a significant decrease ($p \leq 0.05$) in serum concentration in PRL, TSH, Pro hormones in AG and GG groups compared with Wild groups of prolactin gene polymorphism while there is no significant difference in GH , Esr and OXY in AG and GG groups compared with AA group while a significant decrease in TSH in AB and BB compared with AA group of kappa casein gene polymorphism, there was also no significance in GH, Pro, Es, OXY concentration of AB, BB group compared with AA groups.

The current result also revealed no significant difference in Hb, PCV, RBC, MCV, MCHC in all groups of two genotype polymorphism - the study also showed a positive correlation between TSH concentration with prolactin gene polymorphism in AA genotype group and GH with Estrogen hormone in AA group.

In conclusion our result revealed that prolactin gene polymorphism had an effect on the serum Prolactin ,TSH and Progesterone hormones concentration and quantity and quality of milk production while kappa casein gene polymorphism had no effect on casein protein percentage in cross bred dairy cows in Karbala province.

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List of abbreviations

Abbreviation	Abbreviation Meaning
ARMS	Amplification Refractory Mutation System
bPRL	Bovine prolactin
BCS	Body Condition Score
CLA	Conjugated Linoleic Acid
CLDN3	(Claudin 3) is a Protein Coding gene
CLDN4	(Claudin 4) is a Protein Coding gene.
CCP	colloidal calcium phosphate
ES	Estrogen hormone
GH	Growth hormone
IF	inner forward
IR	inner reverse
K-CN	kappa-casein
MG	mammary gland
Oxy	oxytocin hormone
OF	Outer forward
OR	Outer reverse
PRL	prolactin hormone
PRO	progesterone hormone
QTL	Quantity Trait loci
RFLP	Restriction Fragment Length Polymorphism
SREBP1	sterol regulatory element-binding protein 1
S14	thyroid hormone responsive spot 14

TJs	Tight Junction proteins
PCR	Polymerase chain reaction
PRL – G	Prolactin gene
TSH	Thyroid stimulant hormone
JAK2	Janus kinase 2
STAT5	*Signal transducer and activator of transcription 5*
SNP	*Single-nucleotide polymorphism*
ROC	Receiver operating characteristic

Chapter one

Introduction

1.Introduction:

Cow's milk is a source of essential nutrients for newborn calves and as a raw material for human food preparations (Mir *et al*., 2014). The formation and lactose formation of proteins and fats in cow's milk are part of a balanced diet and are responsible for the physicochemical and manufacturing properties of milk and dairy products (Chilliard *et al*., 2000). The production of many hybrid breeds has decreased in our country, and the reasons may lead to a complete loss of some important alleles or genetic variation that would affect future genetic evolution (Al-Shammari, 2015). Milk production and its components is a quantitative trait that is controlled by several genes such as prolactin (PRL) and kappa-casein (Khaizaran, 2014).

Recently, in molecular genetics, newly developed methods of identifying genetic changes in cows have emerged, and breeding is known as selective genes and has been used to determine the quantitative trait point (QTL) in livestock (Bayram *et al*., 2017). The use of polymorphic markers in cattle breeding can make selection more accurate and efficient (Javanmard *et al*., 2005) and There are some local studies that refer to studying genetic changes with milk production in Karbala Governorate, such as the study (Zabeel *et al*., 2019).

Prolactin (PRL), also known as lactotropin, is a polypeptide hormone, secreted mainly by the anterior pituitary gland and It has a molecular weight of appxoximately 22-kDa. It is a single-chain polypeptide of 198 amino acids and is apparently the result of removal of some amino acids. involved in many endocrine activities (Skorupski & Kmieć,2012).

Bovine PRL gene is located on chromosome 23 and comprises five exons and 4 introns. spanning a 10 kb genomic segment and encodes a 199 amino acid mature protein (Uddin *et al*., 2013). Previously several polymorphic sites have been detected within PRL gene and statistically significant associations between PRL variants and milk production traits have been described in dairy cattle (Dong *et al*., 2013). for example, in the bovine genome, a single gene, The bovine prolactin (bPRL) cDNA is 917 nucleotides long and contains a 699-nucleotide open reading frame encoding the prolactin prohormone. The signal peptide contains 30 amino acids, (Marc *et al*., 2000).

Based on its genetic, structural, binding and functional properties, prolactin belongs to the prolactin/growth hormone/placental lactogen family [group I of the helix bundle protein hormones (Wojdak-Maksymiec *et al*., 2008). Significant associations between PRL variants and milk production traits in dairy cattle have been statistically confirmed. (Weikard *et al*., 2005 ; Akyuz *et al*., 2012).

Several genes are involved in milk production. Among them, casein is the main component of the total milk yield. The main milk protein of all mammals in cows is made up of the four casein ins. αs1-casein, αs2-casein, β-casein and k-casein, which account for 80% of the total proteins in milk, as well as the two major whey proteins, α-lactalbumin and β-lactoglobulin 20, which account for 20% of the total proteins. These six previous proteins account for 95% of the total gains in cow's milk (Alipanah *et al*., 2008).

Caseins genes are located within a 200-kb region on chromosome 6 (Ferretti *et al*., 1990; Threadgill and Womack, 1990) and comprised of five exons and four introns (Martin *et al*.,2007). It encoded protein

affecting the amount, composition and technical properties of milk. Eleven genetic variants of CSN3 had been distinguished (Trakovicka *et al*., 2012), the most widely recognized alleles are A and B which are differ in two amino acids at positions 136 (Thr → Ile) and 148 (Asp → Ala). The B allele was found to be associated with thermal resistance, shorter coagulation time, better curdles and micelles of different sizes, which are preferable in cheese making (Schaar *et al*., 1985). The goal of study was to evaluate the genotype and alleles frequency prolactin gene and Kappa casein polymorphism associated with milk traits and hormones levels and hematological parameters and Trace elements in dairy cow in Karbala province.

Aims of the study :

According to the our knowledge this is the second study in Karbala Province about using physiological and molecular assay with milk quantitative trait loci in hybrid dairy cattle , The goal of study was to evaluate the genotype and alleles frequency prolactin gene and Kappa casein polymorphism associated with milk traits and hormones levels and hematological parameters and Trace elements in dairy cow in Karbala province in our country by the fallowing.

- Estimation of serum PRL, GH, TSH, Pro, and OXT hormones in cow cross bred.

- Serum trace elements analysis including Serum Fe, P and Ca in the blood serum.

- Estimation of milk quantity and quality including casein, fat percentage, solid non fat percentage, density, protein percentage, freezing point and lactose percentage.

- Estimation of some hematological parameters. WBC, RBC, Hb, PCV (HCT), MCV, MCH & MCHC.

- Correlation between Prolactin gene polymorphism with PRL, GH, TSH, Pro, Es and OXT hormones

Chapter two

Literature Review

2.Literature Review:

2.1.The best known dairy cow breeds in the world:

2.1.1. Holstein Friesian

2.1.2. Ayrshire

3.1.3 Jersey

4.1.4 Brown Swiss

The specifications, shapes and characteristics of these cows are also under study (Sutarno & Setyawan, 2016)

2.2.Cattle in Iraq

Cattle is the main source of animal protein in Iraq, it is dual purpose cattle producing about 60,000 Mt of meat and 20,000 Mt of milk annually.

Two million heads of cattle are being raised in Iraq , the majority of them (one million) are living in the central area and the rest equally divided in North and South areas (West, 1958 and Spinage, 2003).

2.3.The well-known cattle breeds in Iraq:

2.3.1.AL-Janubi cows

This is living in southern and central areas and used for production of milk while male used for meat production. Morphological and production characters al-Janubi is characterized by the presence of hump and its red color The limbs are long , the skin is soft and the animal is resistant for hot temperature as well as its resistance to endemic diseases (Teodoro *et al*., 1996).

The average weight of the animal is 400 kg at 3-5 years of age, while the quantity of milk 1000 kg during 305 days. It was found that cross breeding of Janubi with Friesian breed can improve its milk and meat production (Madalena *et al*., 1990).

It was also found that improving environmental circumstances of al-janubi can improve its milk production to reach 1350 kg during 200 days, with high percentage of fat (4.8%) , while the first delivery occurs at 2.5 years (Abdel Kariam , 1990) Note that the cows are under study.

2.3.2 Shurabi:

This breed is found in northern area of Iraq, and it is believed that it had been introduced to Iraq from Turkey. Cattle of this breed is characterized by the black color with the presence of longitudinal white lines of hair on back and abdomen. The total body weight of the adult animal reaches to 450 kg with 600 gm a daily increase of body weight and 52% dressing percentage (Yeates *et al*., 2013).

Milk production of Shurabi is lower than 6 kg daily , Thus the breed is considered as beef producing one (AL-Nassir & Ismeal , 2014 ; Ayalew, 2020) as well as that the cows are under study.

2.3.3. Restaki:

These cattle are raised in central and southern areas of Iraq. The breed is better than Janubi in terms of body weight which reaches to 500 kg, and it is better than Karadi breed in both milk and meat production (Abdel Kariam, 1990).

The breed is characterized by its brown to red color or even grey and by its ability to gain weight with 0.688 gm as daily increase of body weight and 54 % dressing percentage. However, its milk production is medium about 3-4 kg/ daily (Mastrangelo *et al*., 2018) as well as that the cows are under study.

2.3.4. Karadi

This breed is living in the northern area of Iraq, which is characterized by its small size and short limbs to enable the animal to move easily in the high land areas. Individuals of this breed are black, red or grey in

color with short horns and small udder of female. Its milk production is low which is not more than 2-3 kg / daily with short lactation period. Moreover, its daily increase of body gain is 400 gm which is lower than janubi and shurabi (AL –Nassir & Ismeal, 2014).

Nowadays, it is common to find cross breeding between the local breeds and Friesian breed which is a successful program to get generations with high performance level and good adaptability to the local environmental circumstances (AL –Nassir & Ismeal, 2014)

2.4.Body morphology and judgement of dairy cows:

The important factor in the selected traits is their economic return, even if they have a simple genetic equivalent or they will take a long time to obtain them.

Udder shape is a fundamental necessity for survival, An important recipe in quantity production as the udder causes the largest number of cows to be excluded comparison with other reasons for exclusion udders with the best production quantity and she is the one who has a strong front and back ligament, and good depth, and a clear incision at the base of the udder indicating strength of the medial suspensory ligament, it has a soft texture that causes the complete collapse of the udder after milking it, this trait has a guest genetic equivalent that is, it responds slowly to selection but it must be pursued in the herd due to the necessity of being related to production (Davis, 2012).

Legs shape is the strength of the legs and their erection is a fundamental thing that cannot be overlooked because it is mainly about cow survival, so there is an important note it is the erection of the lower shin in the hind legs from the back. The more upright it gives the cow the

better strength for its erection on its feet, its fine curvature indicates a lack of calcium in the body Suggesting the weakness of the cow (Castelló, 2016).

Body size an over sized cow is not a good production cow because it is considered less efficient in utilizing the feed you eat as one of the main causes of all other reasons (VandeHaar *et al*., 2016).

Body shape in general, dairy cows are distinguished by their body shape the clear triangle whose top is at the head and its base between the top of the tail and the udder therefore, the dairy cow the closer it will be to this trait the higher the productivity (Adams, 2015).

The shape of the chest and obesity Here we have an important basic characteristic that appears in the triangular chest shape with the overall shape of the cow, and from the ribs of the cow, we can determine the fatness of the cow between a fat cow and a milk cow is a simple criterion hence the last three sides should be noted and be apparently, an increase in their number means wasting, and a decrease in their number means obesity which should be excluded from the characteristics of the milk supply which has an opposite effect in fertility as well as in productivity and reduces the period of survival of the cow (Mammi, 2018).

Head and neck, we talked about the triangular shape of the cow's body and so shall be the head the neck is relatively long for the fattening species not overcrowding with meat (Grandin, 1983).

Milk alert it is the speed of the descent of the milk from the cow when the milking process begins. So that the cow does not delay the normal period of milk It averages 5 minutes (Paulson *et al*., 2015).

Up to the length of stay, that should have ages from three to five productive seasons Then the economist will abandon the cow. From this topic we do not mean by the longevity of the perennial cow but we mean the cow that lives most of its production seasons without problems or risk

to her life with the maximum possible profit An age when the cow is well productive (Garcia *et al*., 2006).

2.5.Milk yield:

Milk yield is affected by genetic and non-genetic factors. Knowledge of these factors is essential for efficient management and accurate estimation of breeding values. For dairy producers, milk yield is one of the main factors that drive economic profitability in dairy farms. Striving to increase milk yield per animal, while decreasing feed and other expenses, can lead to economic gains in farms. In dairy cattle projects, the breeder (farmer) tried to increase the production efficiency of his animals via selections in the last decades (Meredith *et al*., 2012).

In different regions of the world, total milk yield of dairy cattle differ due to many factors such as environment, season, nutrition and differences in genetic makeup of animals, parity, days in milk, year of calving and age of calving (Pirzada, 2011), as well management factors (Javed *et al*., 2000).

2.6.Factors influence milk yield:

2.6.1.Breed:

The significant differences in milk yield and contents including fat and protein may be due to the genotype as well the variations between individuals within a certain dairy breed (Thaller *et al*., 2003). they stated that Holstein cows have the highest volume of milk yield and the highest total production of all major milk components (i.e. fat, protein and lactose). However, there are a lot of variations in milk yield between individuals within a certain dairy breed, While (Maasoom, 1997 ; Latif et

al., 2001) found that, the differences between Friesian and Holstein were not significant.

Ríos-Utrera *et al*., (2013) revealed that Holstein Friesian cows yielded more milk per lactation and per day, respectively, than Brown Swiss cows despite the fact that Holstein Friesians had heavier body weight at calving than Brown Swiss cows. In addition,(Koç, 2007) reported that Holstein Friesians produced more milk/day than Brown Swiss cows in Mediterranean climatic conditions of Turkey.

Gergovska *et al*., (2011) reported that 305-day milk yield of Holstein Friesian cows was higher than that of Brown Swiss cows by 970 kg.

2.6.2.Genetic influence:

Selected genes in selection studies from ruminant breeding of cows, known as Selected genes were used to locate quantitative trait (QTL) in Cattle from cows. The QTL distribution patterns are the most similar to milk the product, milk protein, milk fat and milk fat content (Palombo *et al*., 2018).

Physiological effects on Quantitative characteristics such as increase in the live weight of the animal and milk production as well as a physical link to genes that influence these pathways. The development of any trait in the population depends mainly on Economic profits and the milk farm and its contents are a quantitative trait, it is controlled by many genes (Ebrahimi *et al*., 2015 ; Aytekin & Boztepe, 2013) such as: Prolactin (PRL), Bovine Kappa-Casein (K-CN), and Pituitary transcription factor (pit-1) (Khaizaran, 2014).

2.6.3.Parity:

Several researchers revealed that an increase in parity resulted in an increase in milk yield (Bajwa *et al*., 2004; Musa *et al*., 2005; Hatungumukama *et al*., 2006; and Badri *et al*., 2011). This is a result of the increasing development and size of the udder and the increasing body size over that of the first lactation animal, as well to an increase in body weight, which is associated with enlargement of the digestive system and the mammary gland (Badri *et al*., 2011).

Migose *et al*., (2006) observed that the peak lactation yield in Aryshire crosses is observed in the fourth lactation, and they claimed that milk yield in dairy cows increases with age as a result of a combination of increased body weight and full development of secretory tissues.

2.6.4.Season of calving:

Season of calving has an important impact on productive traits, as the high temperature increases, respiratory rate, severely depresses feed intake and milk yield (Amasaib *et al*., 2011). Hot climate contributes significantly to reduced milk yield indirectly and directly through its effect on feed intake (Nkenwa, 2009).

Milk yield observed to be higher during rainy season due to higher levels of energy, protein and minerals available to the lactating animals during such period (Gimbi, 2006). Milk efficiency was the highest in cows born and calving in the winter and the lowest in cows born and calving in the summer.

It may be wise to decrease the number of heifers and cows calving during summer by regulating breeding programe (Broucek *et al*., 2005). This is because the breed is not heat tolerant during the hot season and during the winter season; there is high voluntary feed intake Turkish

(Koç, 2011). Several studies conducted earlier also revealed to a significant effect of season of calving on milk yield of cows (Jonas *et al*., 2016).

2.6.5.Body weight of dairy cow:

Petrovska and Jonkus , (2014) found a positive relationship between milk yield and live weight and this could be due to the facts that cows with greater live weight can take more feed (Berry *et al*., 2003). Earlier study showed that heavier live weight affects dairy productivity through its effects on extra dietary energy requirements for maintenance and growth (Visscher *et al*., 1994).

Dairy cows have physiological ability of providing nutritional substances from their body tissues by losing "body condition" for about 40 to 100 days (Gergovska *et al*., 2011). They restore the lost body reserves after calving and afterwards (Pryce and Harris, 2006). The interest in this mechanism is the intensive transgeneration genetic selection towards increase of the total milk yield per lactation and at the beginning of lactation (Gergovska *et al*., 2011).

The authors reported that loss of Body Condition Score (BCS) after calving has significant effect on milk yield for 305 days, where the highest is the milk yield of cows with the greatest loss of BCS after calving (Gergovska *et al*., 2011).

2.6.6.Feeding:

A cow's nutritional requirements change with growth and pregnancy And lactation. The most common health-related problems and deaths are in mature dairy products Cows appear within 60 days of calving (Van Saun, 2013).

Good management for The "transition period," which includes the previous month and the month following it from Childbirth, is especially important. When dairy cows feed, they produce from them Nutrition water, energy, protein, fiber, vitamins and minerals. And the grains are The main source of starch in the dairy cow ration (Capper *et al*., 2009).

2.7.Milk production and biosynthesis:

Lactation cycle begins with growth of the breast (Mammogenesis), initiation of milk synthesis and secretion (lactogenesis 1 and lactogenesis 2), established lactation (Galactopoiesis), regression of the breast during and after weaning (involution) (Hartmann et al., 1996) .

Figure (2-1) presents different stages in the lactation cycle starting from Mammogenesis (getting ready for lactation) to lactogenesis (initiation and establishment of lactation) to galactopoiesis (maintenance of lactation) (Truchet & Honvo-Houéto, 2017).

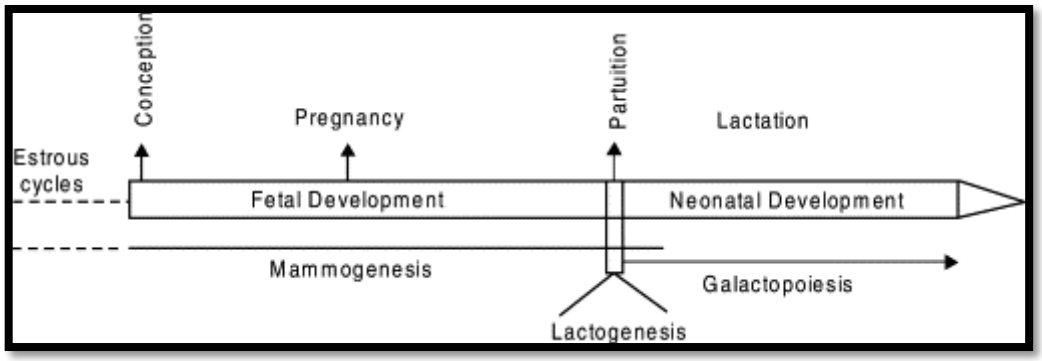

Figure (2-1) presents different stages in the lactation cycle

Mammogenesis is a stage when the mammary gland develops its histological and bio-chemical capacity to synthesize milk. This stage is characterized by increase in number and size of the alveoli, where milk is secreted and stored (Fleming *et al*., 1986) .

Lactogenesis is a stage where the body secretes and produces milk for the fetal. In cows, lactogenesis (referred to as the time when milk 'comes in') starts about 40 hours before the birth and is largely complete within five days. During this stage, a profound and rapid series of changes in the activity of differentiated mamary epithelial cells from a quiescent state to a fully active secretory state occurs (Paintal, 2011).

Lactogenesis has 2 stages: the first stage, lactogenesis 1 occurs by mid pregnancy, when the mammary gland becomes competent to secrete milk. The secretion of milk, however, is held in check by high circulating levels of progesterone and estrogen (Neville, 2006). The second stage, lactogenesis 2 occurs around the time of delivery and is defined as the onset of copious milk secretion (Robinson, 2009).

The hormonal changes during parturition and the subsequent removal of the placenta act as a lactogenic trigger which is necessary for initiation of milk secretion (Neville & Morton, 2001). For the ongoing synthesis and secretion of animal milk, continuous hormonal signals received by the mammary gland (due to stimulation of nipple), relayed to the central nervous system are essential to induce milk secretion (Pillay & Davis, 2020). Milk is secreted more or less continuously into alveolar lumens and stored until the let-down reflex induces milk ejection where it exits through ducts into small sinuses near the areola and then opens directly on the nipple (Madill, 2010 ; Neville, 2006).

When the calf starts suckling at the breast, it causes the stimulation of touch receptors that are densely located around the nipple and areola (Leung & Sauve, 2005). Tactile sensations (created by calves suckling) create impulses that ascend the spinal cord,creating a neuronal pathway between the hypothalamus and pituitary gland which release oxytocin

resulting in the expulsion of stored milk from alveoli into the sinuses through the nipple pore (Huggins, 2017).

Once milk secretion has started, suckling by the calf influences subsequent functioning of the mammary that is jointly controlled by the nervous/endocrine systems through the release of appropriate hormones as well as transfer of nerve impulses (Cahill & Wagner, 2002).

2.8.Proteins and Milk composition:

Milk is a complex biological fluid made out of mainly water, lactose, fat, proteins, organic acids and minerals. It represents a fundamental source of energy, proteins, minerals, and vitamins for young mammals during the first period of their life (Motycka, 2010).

During recent years, the production of milk protein in high-yielding dairy cows has received more emphasis as component pricing based on units of fat and protein has become more established in the dairy factories. In 2011, 61% of skimmed milk collected in Germany was devoted to the protein-dependent production of cheese, milk powder, butter milk, and caseins (Gellrich *et al*., 2014).

However, increases in milk yield are not only associated with increased milk protein production, but in general also with the energy-demanding production of milk fat and lactose in the mammary gland. (Sigl *et al*., 2013), increase in milk protein yields should be accomplished by increasing milk protein concentration with concomitant constant milk yield. Milk protein accounts for approximately 3.2–3.8% of milk, It consists of about 20% whey proteins with major components α-lactalbumin (α LA), β-lactoglobulin (β-LG) and 80% caseins, divided into major subclasses α- (αS1- and αS2-), β-, and κ-casein (-CN), which are arranged in micelles (Mehta, 2015).

Furthermore, minor constituents such as proteolyzed fragments, bovine serum albumin, free amino acids, and immunoglobulins add to the total protein concentration of milk, Caseins, α-LA, and β-LG are synthesized in the epithelial cells of the mammary gland from primary blood constituents, which serve as precursors (Davoodi *et al*., 2016).

For cheese making a higher casein content, particularly higher κ-CN, correlates to increased curd yield, stronger curd firmness, and less casein loss in whey (El-Agamy, 2009). The composition of milk and milk proteins is influenced by many factors. With the increasing age, casein concentration decreases and whey concentration increases, whereas with the increasing lactation, casein concentration increases after its nadir in the 2nd month (Walker *et al*., 2004). Protein composition is also influenced by genotype and diseases (Ayerza, 2009). Amino acid supply of mammary gland is elevated due to feeding higher amounts of rumen-nondegradable protein and roughage which is metabolized by rumen bacteria (Doepel *et al*., 2004 ; Roche, 2006).

2.9.Milk Ejection and the physiological mechanism:

Milk leaves the gland once it is secreted, chiefly due to the interesting neurohormonal reflex known as milk ejection, which takes place within a minute of the start of suckling or milking. This has been well reviewed recently (Crowley, 2011). It is perhaps worth reiterating that all the milk that can be removed by suckling or milking is present as such in the gland at the start and is not, as was once widely thought, partly secreted slowly between milking and partly secreted rapidly in response to stimulation of the teats. It has been shown, (Knight, 2019).

After the initial emptying of the teat and cisterns that slowly fill between the milking, the main bulk of the milk becomes readily available to the milker only after 30 seconds to I minute (Heizer, 2020).

The evidence is almost overwhelming for the view that this rapid transference of milk from the alveoli and small ducts to the large ducts and cisterns is due to the contraction of the mammary myoepithelial cells in response to oxytocin released into the blood stream by the posterior pituitary gland under the influence of afferent nervous impulses from the teats (Cross, 2013).

The basic mechanism of the milk ejection reflex was described by Ely and Peterson in 1941. During the last quarter century the neural pathway of this reflex was traced from mammary gland to brain (Akers, 2016). It was established that supraoptic and paraventricular nuclei in the hypothalamus synthesized oxytocin (Augustine *et al*., 2018) . In addition, oxytocin binds specifically to a protein, neurophysin I, to form granules (Kumar *et al*., 2020). The granules are transported from their site of synthesis in neurons of the supraoptic and paraventricular nuclei to their site of storage in the posterior pituitary (Blevins *et al*., 2004).

Schaeffer *et al*., (2011) reported that stressful stimuli inhibited milk ejection. In intervening years much additional understanding of this phenomenon has been gained (Rushen *et al*., 2007). For example, some stressful stimuli induce release of epinephrine and norepinephrine, which cause vasoconstriction and reduce the amount of oxytocin reaching the myoepithelial cells. Moreover, epinephrine blocks oxytocin binding to myoepithelial cells. Exogenous oxytocin does not overcome this peripheral inhibition of milk ejection.

The most common cause of failure of the milk ejection reflex is associated with stress of milking in early postpartum. This stress sometimes inhibits release of oxytocin from the posterior pituitary gland (Tancin *et al*., 2001).

2.10.Bovine mammary gland development (Morphogenesis)

The mammary gland undergoes dynamic morphological changes over the lifetime of female mammals. At birth, bovine mammary parenchyma consists of a rudimentary duct network connected to a small cisternal cavity. At the onset of puberty, the mammary rudiment develops and starts to expand into the stroma upon stimulation by the ovarian steroid hormones, including estradiol and progesterone, and by growth factors (Yart *et al*., 2014). Ductal elongation occurs through the growth, development, and subsequent extension of terminal ductal lobular units (TDLU) the mammary parenchyma of bovines develops into a compact, highly arborescent, parenchymal mass surrounded by a dense matrix of connective tissue (Akers, 2017).

Bovine mammary TDLUs initially consist of solid cords of epithelial cells that penetrate into the stroma. As these cords extend into the mammary fat pad, lateral outgrowths emerge. This parenchymal development continues through puberty, until the mammary fat pad becomes filled. In addition, during gestation, the tissue continues its differentiation with the formation of lobulo-alveolar structures and the maturation of TDLUs in response to circulating hormones, notably prolactin. At the end of its development, the mammary epithelium has the appearance of an elaborate tree of ducts and alveoli. After parturition, the alveolar epithelium starts to be fully functional, with mammary epithelial

cells secreting milk proteins into the lumen of the alveoli for lactation (McBryan and Howlin, 2017).

The epithelial hierarchy can be described as a pyramidal setup of the epithelial cell populations with stem cells at the apex and differentiated mature cells at the base of the pyramid. Between these two cell populations are the multiple progenitors that originate from the division and activation of stem cells and that progressively differentiate into mature cell lineages. Of note, the mammary 63 structures are described as being composed of two major lineages: the luminal and basal cells, the latter including the myoepithelial cells (Martignani *et al*., 2009).

Luminal and basal cells can be distinguished by either their location in the epithelial structure or their protein expression profiles. Cells of these two lineages are considered immature during development as compared to the differentiated (mature) cells that constitute the functional secretory tissue. In contrast, in bovines, only a few groups have attempted to elucidate the epithelial hierarchy via the identification of progenitor/stem cell populations (Rauner and Barash, 2012).

Capuco *et al*., (2012) A better understanding of the epithelial hierarchy at each developmental stage is therefore a prerequisite for the optimization of lactation in cows.

2.11.Bovine mammary gland development and its Hormones (Mammogenesis):

Estrogens caused mammary duct growth whereas progestins, especially when combined with estrogens, promoted lobule-alveolar development Anumber of studies implicated anterior pituitary hormones in control of mammary growth (Cowie, 2020).

Estrogens and growth hormone promote mammary duct growth, whereas progestins and prolactin stimulate lobule-alveolar development. Maximal mammary development was achieved with these four hormones plus glucocorticoids (Stiening, 2005). Great strides were made in elucidation of mechanisms whereby ovarian steroids affect mammary growth. For example, estrogen and progesterone reduce time for mammary DNA synthesis (Trott *et al*., 2008). Whereas only progesterone induces DNA synthesis along ductular walls (Finucane *et al*., 2008).

Found specific estrogen receptors in mammary tissue Muldoon (Prossnitz *et al*., 2007) showed that estrogen receptors first appear in cows mammary tissue near puberty and that these receptors increase proportionately to increasing weight of the tissue As might be expected, progesterone is bound to its receptor in mammary tissue of virgins and during pregnancy, but the receptor disappears during lactation and reappears during involution tissue coincide with mammary growth responses.

Hypothalamic hormones in regulation of anterior pituitary function was established firmly (Miller, 2018). Prolactin secretion is inhibited tonically as a result of a factor(s) secreted from the hypothalamus into portal vessels of the pituitary stalk and delivered to the anterior pituitary (Le Tissier *et al*., 2017). This important source of mammogenic hormones. Indeed, a hormone unique to placenta that possesses structural and immunological homologies with prolactin and growth hormone has been identified in a variety of species (Forsyth & Wallis. 2002). considerable lobule-alveolar development (Mixner *et al*., 1942).

In most species, secretion of placental lactogen commences about midpregnancy and remains elevated until parturition. Thus, it is likely

that placental lactogen synergizes with estrogens, progestins, prolactin, and growth hormone to cause mammary development during normal gestation. Increased secretion of placental lactogen may account for greater mammary development as number and weights of placentas increase in several species, including goats (Neville, 2006). Mammotrophic hormones after mid-gestation subsequently (Hiew, 2014).

These data, therefore, support the concept that prolactin is essential for full lactogenesis in cattle (Akers, 2006). Prolactin receptors in mammary gland increase in parallel with increased secretion of prolactin in the periparturient period coincident with secretion of copious quantities of milk (Akers, 2016). Collectively The role of growth hormone in lactogenesis appears to be one of synergism with prolactin, and adrenocorticotropin. Although a surge in secretion of growth hormone occurs coincident with parturition (Cowie, 2020). For hormones to exert their action, they usually bind to specific high affinity receptors in the mammary cell (Walther *et al*., 2005).

In the alveolar cell cortisol induces differentiation of the rough endoplasmic reticulum and Golgi apparatus (Squires, 2010).glucocorticoids bind to specific receptors within the mammary cell. Moreover, progesterone blocks binding of cortisol effectively at the glucocorticoid binding site (Borski, 2000). Progesterone binds to a progesterone receptor in mammary tissue (Rekawiecki *et al*., 2011). increased clearance and secretion rates of prolactin are greater in early than late lactation and are associated positively with increased milk production in dairy cattle (Davis *et al*., 2021) Also, glucocorticoids are taken up from the blood at milking, and they become bound to specific receptors in mammary tissue (Cassoni *et al*., 2001).

2.12.Role of hormones in dairy cows:

2.12.1Growth Hormone (GH) :

Somatotropin (ST), also known as Growth Hormone (GH), is a small protein molecule that contains 191 amino acids in a single chain is a 22 kD peptide hormone secreted by the anterior pituitary gland. Bovine growth hormone (bGH) , or Somatotropin GH is secreted by somatotropes, the most numerous cells in the anterior pituitary (Jiang et al., 2012). the pituitary produces at least a thousand times as much GH as any other hormone. that influences several complex physiological processes, including body growth and tissue metabolism and modulation of embryo metabolism during bovine embryogenesis (Sanders & Harvey, 2004).

It promotes increased sizes of the cells and increased mitosis, with development of greater numbers of cells and specific differentiation of certain types of cells such as bone growth cells and early muscle cells. In mammary tissues, but has widespread effects on the body, especially on cartilage, bone, muscle, and fat (Pepper *et al*., 2007).

Its effects include an increased uptake of nutrients and an augmented secretory cell activity and survival capability, which lead to enhanced milk synthesis. In the last two decades a number of scientific efforts have been targeted towards the development of biotechnologies with the aim of increasing the efficiency of milk yield, especially in cattle (Tizard et al., 2016). This trend led to the production of recombinant bovine somatotropin (rbST), developed to increase milk production efficiency in dairy cows . bGH increases lactation milk yield by altering the lactation curve by increasing the number of cells (hyperplasia) and increasing the size of cell (hypertrophy) (Lucy *et al*., 2001) .

The galactopoietic effect of bGH can be explained by its direct involvement in many tissues and metabolism. Recombinant bovine somatotropin (rbST) on milk yield,milk composition (fat and protein), milk somatic cell count, and body condition score (BCS) among dairy cattle (VanBaale *et al*., 2005 ; Park *et al*., 2002).

2.12.2.Prolactin hormone (PRL) :

Prolactin (PRL), also known as lactotropin, is a polypeptide hormone, secreted mainly by the anterior pituitary gland and It has a molecular weight of appxoximately 22-kDa. It is a single-chain polypeptide of 198 amino acids and is apparently the result of removal of some amino acids. involved in many endocrine activities (Skorupski & Kmieć, 2012).

A protein best known for its role in enabling cows usually females, to produce milk. It is influential in over 300 separate processes in various vertebrates, Prolactin is secreted from specialized cells of the anterior pituitary gland in response to eating, mating, ,ovulation (Clarke & Bern, 2012) .

It is secreted heavily in pulses in between these events. The structure of prolactin is similar to that of growth hormone and placental lactogen. Additionally, PRL plays a crucial role in signal transmission during the processes of milk production and exerts its physiological effects to induce lactation by acting through the dimerization of PRLR (Freeman *et al*., 2000 ; Forsyth & Wallis, 2002).

Bovine PRL gene is located on chromosome 23 and comprises five exons spanning a 10 kb genomic segment and encodes a 199 amino acid mature protein (Uddin *et al*., 2013). Previously several polymorphic sites have been detected within PRL gene and statistically significant

associations between PRL variants and milk production traits have been described in dairy cattle (Dong *et al*., 2013)

Significant associations between PRL variants and milk production traits in dairy cattle have been statistically confirmed. prolactin is a peptide hormone, encoded by the PRL gene (Weikard *et al*., 2005 ; Akyuz *et al*., 2012). that was originally named for its ability to promote lactation in response to the suckling stimulus of hungry. In mammals, prolactin is associated with milk production Prolactin also acts in a cytokine-like manner and as an important regulator of the immune system (Dobolyi *et al*., 2020). Binding to cytokine-like receptors. We now know that prolactin is not as simple appears in a multiplicity of posttranslational forms ranging from size variants to chemical modifications such as phosphorylation or glycosylation (Melmed & Kleinberg, 2003).

Prolactin-releasing stimuli not only include the nursing stimulus, but light, audition, olfaction, and stress can serve a stimulatory role. Finally, although it is well known that dopamine of hypothalamic origin provides inhibitory control over the secretion of prolactin,other factors within the brain, pituitary gland, and peripheral organs have been shown to inhibit or stimulate prolactin secretion as well (Ben-Jonathan, & Hnasko, 2001; Fitzgerald & Dinan, 2008).

It influences hematopoiesis and angiogenesis, and is involved in the regulation of blood clotting through several pathways. The hormone acts in endocrine, autocrine and paracrine manner through the prolactin receptor and numerous cytokine receptors Pituitary prolactin secretion is regulated by endocrine neurons in the hypothalamus (Ahima & Flier, 2000 ; Fruhbeck *et al*., 2001).

The most important of these are the neurosecretory tuberoinfundibulum (TIDA) neurons of the arcuate nucleus that secrete dopamine (aka Prolactin Inhibitory Hormone) to act on the D2 receptors of lactotrophs, causing inhibition of prolactin secretion (Horjales-Araújo, 2010 ; Dussor *et al*., 2018). thyrotropin-releasing factor (thyrotropin-releasing hormone) has a stimulatory effect on prolactin release, however prolactin is the only adenohypophyseal hormone whose principal control is inhibitory (Flückiger *et al*., 2012) .

2.12.3.Oxytocin (OXY) :

Oxytocin (OXY) is a peptide neurohypophysial hormone made up of nine amino acids (a nonapeptide) in the following sequence: cysteine, tyrosine, isoleucine, glutamine, asparagines, cysteine, praline, leucine, and glycine. It is produced primarily in two discrete locations in the brains of all male and female mammals and plays an important role in milk letdown, the contraction of the smooth uterine muscles during the birthing process, and various maternal behaviors (Emea, 2001, Epa, 2005).

The name “oxytocin” means “rapid birth,” due to its ability to contract the pregnant uterus (Gimpl and Fahrenholz, 2001). that is best known for its ability to milk ejection in mammals. Under natural conditions, it is released from the posterior pituitary and causes contraction of the muscle cells surrounding the milk alveoli in mammary gland for milk let-down. In bovine mammary gland about 80% of milk is stored in the alveoli and is transferred into the milk cistern by the milk ejection reflex (Bruckmaier *et al*., 1994 ; Prakash *et al*., 2009 & Knight, 2019).

Milk ejection is a neuroendocrine reflex; where, OXY released from the pituitary in response to tactile teat stimulation, causes myoepithelial

cells surrounding the alveoli to contract, forcing milk stored in the alveoli into the mammary ducts and gland cistern (Ferneborg, 2016).(Knight, 2019 & Bruckmaier *et al*., 1994). so every time during the milking of a cow/buffalo there is a natural release of minute quantities ranging fromaround (15 to 90 pgmL−1) of this hormone into blood circulation only for a fewminutes. The other important role of OXY is stimulation of uterine concentration during parturition or labor (Prakash *et al*., 2009).

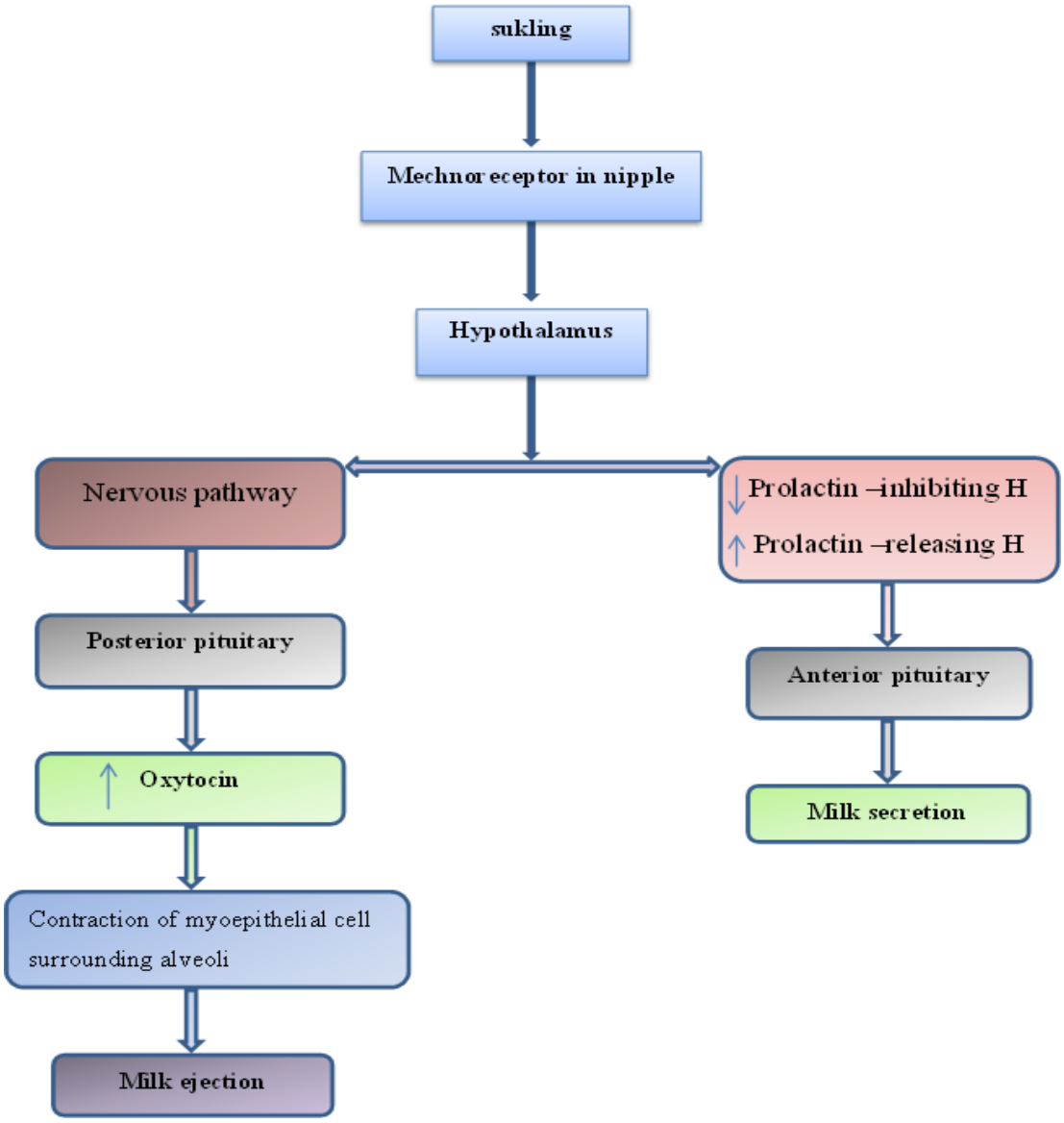

Figure (2-2) schematic diagram of suckling reflexes (Tancin, & Bruckmaier, 2001).

2.13.Hypothalamic-pituitary axis reflex:

The hypothalamus is the integrator of the neuroendocrine system monitoring neurological, chemical, and hormonal inputs, comparing these to physiological set points (electrolyte and fluid balance, body temperature, blood pressure, and body weight) and responding both neurologically and through hormone secretion to restore homeostasis (Rohrbasser *et al*., 2018). This involves the complex integration of positive and negative feedback loops and synaptic inputs from other brain areas and from the autonomic nervous system. Hypothalamic neuropeptides are also secreted in regions of the brain out with the hypothalamus where they modulate and coordinate behavior to complement their hormonal actions (Ferguson *et al*., 2008).

The secretions of the pituitary gland are as follows. The anterior lobe synthesizes and secretes six principal hormones: follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), growth hormone (GH), and prolactin (PRL). the first five of these are tropic, or trophic,9 hormones— pituitary hormones that stimulate endocrine cells elsewhere to release their own hormones important (Peper *et al*., 2010).

More specifically, the first two are called gonadotropins because their target organs are the gonads. Seven of them, and travel through the portal system and regulate side by side and are so closely joined that they look like a single gland. the activities of the anterior pituitary. Five of these are releasing hormones that stimulate the anterior pituitary to secrete its hormones, and two are inhibiting hormones that suppress pituitary secretion. Most of these hypothalamic hormones control the release of just one anterior pituitary hormone (Barkhoudarian & Kelly, 2017).

The hypothalamic hormones include thyrotropin-releasing hormone and Gonadotropin-releasing hormone, however, controls the release of both follicle-stimulating hormone and luteinizing hormone , growth hormone-releasing hormone, somatostatin, corticotrophin-releasing hormone, dopamine, and The other two hypothalamic hormones are secreted by way of the posterior pituitary (Rispoli & Nett, 2005).

These are oxytocin (OXY), and Antidiuretic Hormone (ADH). OT is produced mainly by neurons in the paraventricular nuclei of the hypothalamus, so-called because they lie in the walls of the third ventricle (the nuclei are paired right and left). ADH is produced mainly by the supraoptic nuclei, so-called because they lie just above the optic chiasm on each side (Jacobson *et al*., 2018).

Each nucleus also produces smaller quantities of the other hormone. ,and AVP. The set points remain stable from day to day through homeostasis, the coordinated integration of the classic neuroendocrine pathways with the autonomic and central nervous systems. and The anterior pituitary gland plays a critical role in homeostasis by integrating complex peripheral signals from the hypothalamus and other peripheral organs, intrapituitary signals, and external stimuli to regulate release of anterior pituitary hormones into the peripheral circulation (Rohrbasser *et al*., 2018).

The classical feedback mechanisms of the hypothalamic pituitary axis and its target organs were described many years ago. In more recent years, our understanding of the role of intrapituitary regulators of pituitary cell growth, apoptosis, hormone secretion, and hormone release has expanded rapidly, to give finer detail of the complex mechanisms underlying the release of anterior pituitary hormones. The hormonal

relationship between the hypothalamus, pituitary, and a more remote endocrinegland is called an axis (Musumeci *et al*., 2015).

There are three such axes, the hypothalamic-pituitary-gonadal axis involving GnRH, FSH, and LH, the hypothalamic-pituitary-thyroid axis involving TRH and TSH, and the hypothalamicpituitary- adrenal axis involving CRH and ACTH . The pars intermedia is absent from the adult human pituitary, but is present in other animals and the human fetus. In other species, it secretes melanocytestimulating hormone (MSH), which influences pigmentation of the skin, hair, or feathers. Humans, however, apparently produce no circulating MSH. Some anterior pituitary cells derived from the pars intermedia produce a large polypeptide called proopiomelanocortin (POMC) (Tran *et al*., 2021).

2.14.Prolactin Gene:

2.14.1.Primary Structure, and Species Specificity:

Based on its genetic, structural, binding and functional properties, prolactin belongs to the prolactin/growth hormone/placental lactogen family [group I of the helix bundle protein hormones (Wojdak-Maksymiec *et al*., 2008). genes encoding prolactin, growth hormone, and placental lactogen evolved from a common ancestral gene by gene duplication (Forsyth & Wallis, 2002).

The divergence of the prolactin and growth hormone lineages occurred ;400 million years ago (Cooke *et al*., 1980 and Cooke *et al*., 1981) (Takahashi *et al*., 2013). for example, in the bovine genome, a single gene, found on chromosome 20, encodes prolactin. The prolactin gene is about 10 kb in size and is composed of 5 exons and 4 introns. The bovine prolactin (bPRL) cDNA is 917 nucleotides long and contains a 699-nucleotide open reading frame encoding the prolactin prohormone. The

signal peptide contains 30 amino acids, thus the mature bovine prolactin is composed of 199 amino acids (Marc *et al*., 2000).

Cao *et al*., (2002) had cloned the whole sequence of bovine PRL gene cDNA (GenBank serial number: AF426315) and proved the biologic transcription activity of bovine PRL gene. To study the effect of bovine PRL gene on dairy milk traits, we chose bovine PRL gene as a candidate gene for milk traits and detected 5'-regulatory region of it, analyzed the relationship between different genotypes and milk traits (Li *et al*., 2006).

The aim was to find genetic markers highly correlated with milk traits for marker assisted selection (MAS) of dairy.Transcription of the prolactin gene is regulated by two independent promoter regions. The proximal 5,000-bp region directs pituitary-specific expression (Berwaer *et al*., 1991), while a more upstream promoter region is responsible for extrapituitary expression (Berwaer *et al*., 1994). the prolactin molecule is arranged in a single chain of amino acids with three intramolecular disulfide bonds between six cysteine residues (Cys4-Cys11, Cys58-Cys174, and Cys191-Cys199 in humans) (Qian et al., 2018) (Cooke *et al*., 1981).

The sequence homology can vary from the striking 97% among primates to as low as 56% between primates and rodents (Sinha, 1995). in rats (Cooke *et al*., 1980) and mice (Kohmoto *et al*., 1984), pituitary prolactin consists of 197 amino acids, whereas in sheep (Kaplan & Boztepe, 2010, June) (LI *et al*., 1970), pigs (LI, 1976), cattle (Dybus *et al*., 2005)(Wallis, 1974), and humans (Shome And Parlow, 1977) it consists of 199 amino acids with a molecular mass of ;23,000 Da. The gene under current study.

2.14.2.Secondary and Tertiary Structure of Prolactin:

Studies on the secondary structure of prolactin have shown that 50% of the amino acid chain is arranged in a-helices, while the rest of it forms loops (Bewley And Li, 1972 ; Baumbach *et al*., 2020). although it was predicted earlier (Niall *et al*., 1971), there are still no direct data about the three-dimensional structure of prolactin.

The tertiary structure of prolactin was predicted by homology modeling approach (Goffin *et al*., 1995) (López *et al*., 2013), based on the structural similarities between prolactin and other helix bundle proteins, especially growth hormone. According to the current three-dimensional model, prolactin contains four long a-helices arranged in antiparallel fashion (Abdel-Meguid *et al*., 1987 and De Vos *et al*., 1992) The gene is also under study.

2.15.Bovine kappa-casein gene:

Many genes are involved in milk production. Among them, caseins are the essential constituents of total milk proteins. In bovines, The essential milk proteins of all mammals comprise of the four caseins; αs1-casein, αs2-casein, β-casein and k-casein which represents 80% of total milk proteins as well as the two major whey protein, α-lactoalbumin and β-lactoglobulin which represent 20% of the total proteins. These previous six proteins represent 95% of total proteins in bovine milk (Alipanah *et al*., 2008).

The candidate gene methodology is a standout amongst the most imperative technique to investigate genetic markers Linked to production traits and to determine the polymorphisms in the protein coding regions. Recently, genetic marker research applied to animal breeding and production is concentrated mainly on analyzing mutations located within

candidate genes and their association with economically important traits (Oikonomou *et al*., 2011). caseins genes are located within a 200-kb region on chromosome 6 (Ferretti *et al*., 1990; Threadgill and Womack, 1990) and comprised of five exons and four introns (Martin *et al*.,2007).

It encoded protein affecting the amount, composition and technical properties of milk. Eleven genetic variants of CSN3 had been distinguished (Trakovicka *et al*., 2012), the most widely recognized alleles are A and B which are differ in two amino acids at positions 136 (Thr → Ile) and 148 (Asp → Ala). The B allele was found to be associated with thermal resistance, shorter coagulation time, better curdles and micelles of different sizes, which are preferable in cheese making (Schaar *et al*., 1985).

Genetic variants of bovine kappa-casein gene are associated with protein content of milk and have a significant influence on rennet clotting time, firmness and cheese yield of milk with a superiority of milk from cows with K-CN BB compared to K-CN AA milk (Tucker., 1981; Collier *et al*., 1984).

This feature suggests that this locus might be used as a genetic marker for milk production. The target of the current study was to analyze kappa casein gene using PCR–RFLP followed by DNA sequencing and to explore conceivable relationship with milk production characteristics in Holstein Friesian cattle (Awad *et al*., 2016) The gene is also under study.

Chapter Three

Materials and Methods

3.Materials and Methods

3.1.Materials:

The Devices and Instrument and materials which used in this study:

Table 3.1 The Instrument and tools which used in this study.

No.	Instrument	Company	Origin
1	Anticoagulant tube (EDTA tube)	(AFMA-Dispo)	(Japan)
2	Centrifuge	Memmert	Germany
3	EKOMILK Ultrasonic milk analyzers	EON TRADING	USA
4	Eppendrof centrifuge (cooling centrifuge)	Fisons	England
5	ELISA - Reader and washer	Biotek	USA
6	Gel electrophoresis	Clever	USA
7	Hematological outoanalyzer	Genex	USA-florida
8	Milk bucket (container)	Al-rawin	China
9	Micrcenterfuge tubes	Eppendrof	Germany
10	Micropipettes (5-50 , 10-100, 0.5-10 µl)	Hirschmann	Germany
11	PCR system (Thermocycler)	Clever	USA
12	PCR tubes 50µl	Hirschmann	Germany
13	UV-transilluminator	Clever	USA
14	Vortex	Gemmy	Twain

3.2.Elisa Kits And Chemical Materials :

Table 3.2 Kits and chemical materials which used in this study with companies and countries of origin.

No.	Kits	Company	Origin
1	Bovine growth hormone ELISA Kit	Sunlong	China
2	Bovine TSH ELISA Kit	Sunlong	China
3	Bovine Prolactin hormone ELISA Kit	Sunlong	China
4	Bovine Progesterone ELISA	Sunlong	China
5	Bovine Estrogen ELISA	Sunlong	China
6	Bovine Oxytocin ELISA Kit	Sunlong	China
7	Normal saline	Himedia	India
8	Ethanol 99%	Himedia	India
9	Agarose	Bioneer	Korea
10	Ethidium Bromide	Bioneer	Korea
11	Tris - Borate - EDTA (TBE) buffer	Bioneer	Korea

3.3.DNA Extraction:

3.3.1.DNA Extraction Kit:

Table 3.3 DNA Extraction Kit contents:

No.	Mini-DNA Extraction kit components	Company	Origin
	- Buffer CL - Buffer BL - Buffer WA - Buffer WB (Concentration) - Buffer CE - Spin Column (Green color) & Collection tube - RNase A, lyophilized - Proteinase K, lyophilized - Manual	Intron	Korea

3.3.2.Primers:

ARMS- PCR primers for detection Prolactin gene polymorphism and another RFLP- PCR primers for Kappa casein gene polymorphism. These primers was provided from Macrogen company, Korea as following Table (3.4 and 3.5).

Table 3.4 Nucleotides primers for detection Prolactin gene polymorphism.

Type of gene	Primer	Sequence 5'-3'	Amplicon	References
Prolactin gene	IF	ACCCTCTGTATCACCTAGTCACCGAGTTG	164 bp	Present study
	IR	CATCTGGGGCTCCTTTCATACCCAGT	191 bp	
	OF	GGTCAATCACTCTGAGCAAAAATCACATG	300 bp	
	OR	AATAGCAAGGAAGCTTTCATGAAGCTGC		

Table 3.5 Nucleotides primers for detection Kappa casein gene polymorphism.

Type of gene	Primer	Sequence 5'-3'	Amplicon	References
Kappa casein gene	F	TGTGCTGAGTAGGTATCCTAGTTATG	426 bp	(Muhammad *et al.*, 2008)
	R	GCGTTGTCTTCTTTGATGTCTCCTTAG		

3.4. Experimental Design

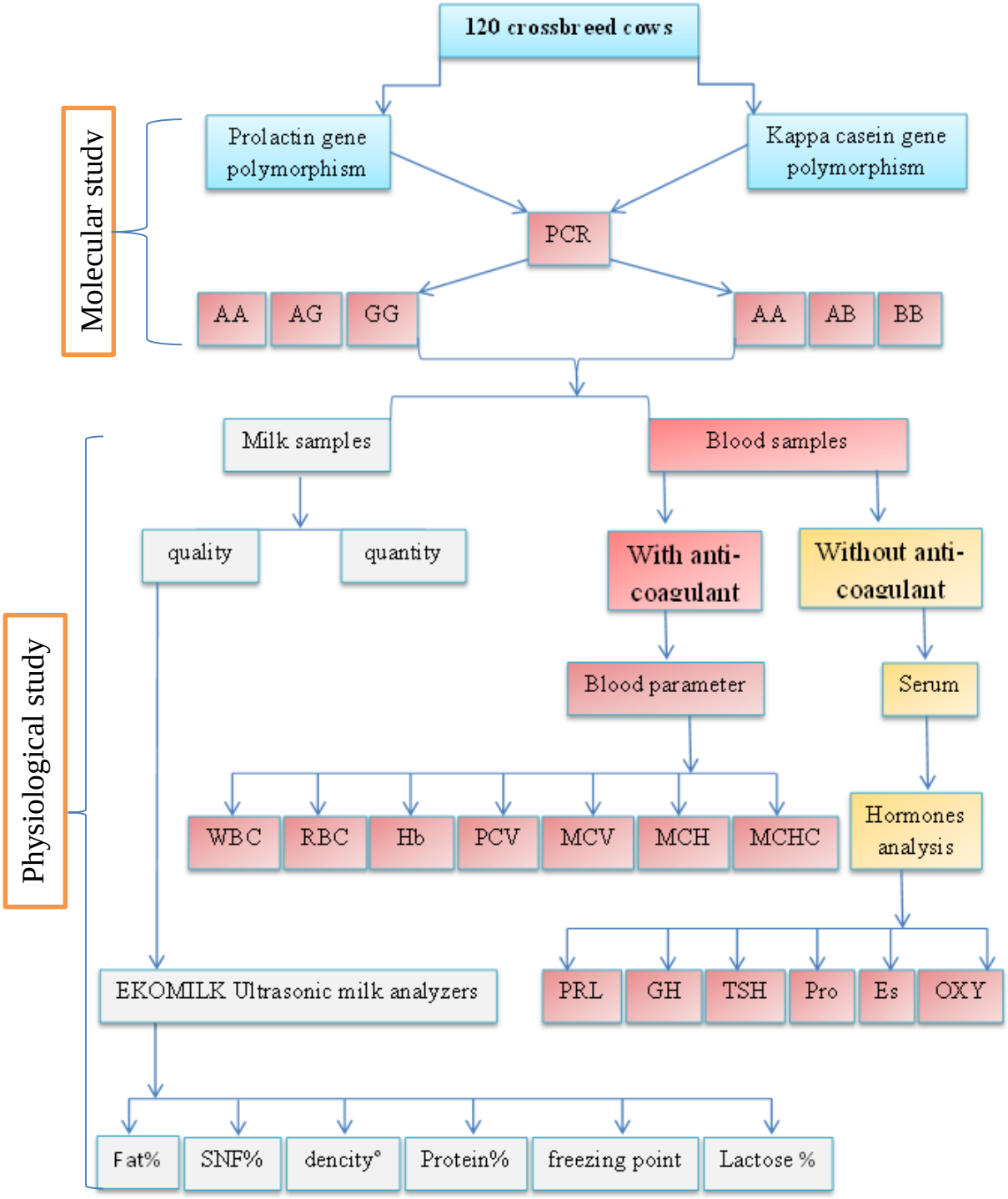

Figure (3-1) Experimental Design

3.5.Animal sample collection:

2.5.1.Animal choosing:

One hundred twenty apparently healthy dairy crossbred cows were used in this study , their age ranged from (4-6) years ,and weight ranged from (278 – 305 KG) in mid stage (40-120) days of lactation, according to heart girth equation (Wangchuk et al., 2018) to the obtained from unorganized fields of rural area in different region of Karbala city during the period from (December 2020 - May 2021) appendix 1.

3.5.2.Collection of Blood Samples: Ten milliliters of blood have been taken from all animals from jugular vein by sterile disposable syringe and collected in three tubes for the following analysis .

-DNA extraction : 3 ml of blood was put into EDTA tubes for prolactin and Kappa casein genes polymorphism analysis appendix 3.

-Hormonal analysis : 4 ml of blood was put in a gel tube for some hormones estimation in cattle serum which including PRL, OXY ,TSH , GH ,ES,PRO appendix 4 .

-Hematological analysis : 3ml put into EDTA tubes used for blood analysis ,including WBC, RBC, HB, HCT, MCV, MCH & MCHC.

3.5.3.Collection of Milk Samples:

30ml of milk have been taken from lactation cattle and collected in sterile disposable cup and stored in ice box at (4C) until the milk was analyzed by (EKOMILK Ultrasonic milk analyzers) in public health laboratory in veterinary medicine college of AL-Kuffa university for determination of protein ,fat, density, freezing point, non solid fat, lactose. appendix 2

3.6.methodes:

3.6.1.Hormonal analysis:

The serum gel tubes were centrifuged at 2500 xg for 10 minutes, separated and divided into two aliquots and stored at (–20 C) until time of use for hormones estimation (GH, TSH, PRL, PRO, Es and OXY hormones) by using Enzyme linked ImmunoSorbent Assay **(ELISA),** According formation of kits appendix 4 (1,2,3,4,5,6).

3.6.2.Hematological analysis:

The hematological parameters analysis was done in Laboratory of Research and Studies / Collage of Veterinary Medicine University of Karbala by using Hematology autoanalyzer made in Genex company ,according the manufacture company. The instrument can measure and calculate 22 different parameter. These instruments used two reagent only (Dilute and Lyse) and Maintenance reagent (Probe cleanser only) and it has a mechanical picture inside with thermal paper. The hematological parameters estimated by instrument were (WBC, RBC, Hb, PCV, MCV, MCH & MCHC**).**

3.6.3.Milk analysis:

Information on milk yield were gained from unorganized farms for each cow (Appendeix 5) with exclusion of cows which have some criteria mentioned later. whole morning and evening milk were collected during milking into a sterile bucket and the milk weight was determined with a spring scale.10ml of milk samples were measured by EKOMILK Ultrasonic milk analyzers, Ultrasonic milk analyzer system determined the milk components percentage were run to obtain an average of parameter by infrared spectrum for milk samples according to the value

of absorbance a each wavelength the concentrations of fat, SNF, density, protein, freezing point and lactose.

3.6.4.Genetic Analysis:

3.6.4.1.DNA Extraction:

3 ml of blood in EDTA tubes was used to extract DNA by using Mini DNA extraction Kit with whole Blood Protocol (Appendix 3) according to the manufacturing company.

3.6.4.2.PCR protocols:

The PCR optimization for prolactin and kappa casein gens were clarified in the following Table 3.6.

PCR protocol		Temperature	Time	References
Initial Denaturation	PRL	95C	5min.	Prolactin Polymorphism Present study
	Kappa C	95C	5min	
Denaturation	PRL	95C	35 Sec	
	Kappa C	95C	30 Sec	
Annealing	PRL	58C	55 sec	Muhammad et al., 2008
	Kappa C	55 C	50 sec	
Extension	PRL	72 C	1 min	
	Kappa C	72C	1 min	
Final Extension	PRL	72 C	7 min	
	Kappa C	72C	5 min	-

The amplification products were held through 1.5% agarose gel stained with and ethidium bromide, the procedure involved all the four different primers in one reaction tube, the reaction tube detected the mutant-type (denoted by the letters B) and the wild-type denoted as (A)

for Kappa Casein polymorphism, while the mutant-type of Prolactin gene polymorphism(denoted by the letters G) and the wild-type denoted as(A).

3.6.4.3.Amplification Refractory Mutation System (ARMS):

Milk Prolactin gene sequences were designed Prolactin gene sequences and annotation by using primer 3 plus, (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi),

The reactions of ARMS-PCR were optimized for 25 µl final volume using 50-100 ng genomic DNA, 200µM each dNTP, 15 mM MgCl2; 1µl from 7 Picomole of each primer (four different primers), 5x Green Go Taq Reaction buffer, 0.5 U of GoTaq DNA Polymerase (Promega) and completed with nuclease-free water.

3.6.4.4.RFLP-PCR Technique

All positive and negative results prescribed by RFLP- PCR were used in RFLP-PCR technique as performing the detection of Kappa casein . This method was carried out according to (Muhammed et al., 2008) and follows :

3.6.4.5.PCR master mix preparation

PCR master mix was prepared by using (AccuPower PCR PreMix Kit) and this master mix done according to company instructions as in the table below for each gene : **Table 3.7 PCR master mix preparation**

PCR Master mix	Volume
DNA template	5µl
Forward primer (10pmol)	1.5µl
Reveres primer (10pmol)	1.5µl
PCR water	12µl
Total volume	20µl

After that, these PCR master mix components that mentioned in the table above were placed in standard AccuPower PCR PreMix Kit that contains all other components which needed PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and loading dye). Then, all the PCR tubes were transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermocycler (Mygene. Korea).

3.6.4.6.RFLP-PCR mix preparation

RFLP-PCR mix for Kappa casein gene polymorphism was prepared by using two restriction enzymes; *Hinf1* restriction enzyme (New England Bio labs. UK) for detection Kappa casein , this master mix was done independeniy according to company instructions as in the following Table 3.8.

Table 3.8 RFLP-PCR master mix:

RFLP-PCR Master mix	Volume
PCR product	10µl
Restriction enzyme buffer 10X	2 µl
Restriction enzyme (10 unit)	1 µl
Free nuclease water	7 µl
Total volume	20 µl

After that, this master mix was placed in Exispin vortex centrifuge at 3000rpm for 2 minutes, then transferred into incubation at 37°Cfor overnight. After that, RFLP-PCR product was analysed by 1.5% agarose gel electrophoresis methods mentioned in PCR product analysis. The

PCR Product was digested by *HinfI* restriction enzyme into (AA=326/100/27, BB=426/27 and AB=426/326/100/27).

3.7.Exclusion criteria

1- Cows with ages more than 6 years and less than 4 years .

2-Pregnant cows.

3-Post medical interference .

4-Early and late stages of lactation .

5-Acut and chronic mastitis .

6- Hot enviromental condition .

3.8.Body weight measurement:

A prediction equation for cattle body weight was estimated and calculated

according to the following formula :

W=(L.G2)/300

W: is body weight of animal in pounds

L: is the length from the point of shoulder to pin bone measured in inches

G: is the chest girth of the animal in inches.

The final weight was converted from pound to kg by multiplication in 0.4536) (Wangchuk *et al*., 2018) . appendix 6.

3.9.Statistical analysis:

All the data were written in Excel sheet , the experience was designed according to the Complete Randomly Design(CRD) ,the results were analyzed using Statistical Software (SAS Institute 2002 Ver. 9) and arithmetic averages were measured on Duncan Multidimensional scale (Duncun,1955).

The Frequencies of the Prolactin gene polymorphisms AA, AG and GG and Kappa casein gene Polymorphisms AA, AB. BB were expressed

in numbers and percentages for wild-type, heterozygosity genotypes polymorphism (mutation). $\chi 2$ test was used to evaluate consistency of genotype distributions with Hardy-Weinberg equilibrium. Appendix 7

Chapter Four

Results

4.Results :

4.1. The number of cows with polymorphism.

from the prolactin genotype variants obtained through the ARMS-PCR technique, the genotype GG had the greatest frequency, it was found out that 56 cattle from 120 dairy breed cow when among of Kappa casein Polymorphism techniques, through the RFLP-PCR technique with the Hinf1 endonuclease, a total of 120 dairy cattle 74 cow have been carry AA genotype and recorded as great frequency and this study is the first of its kind in Iraq Table (4-1).

Table 4-1 : number of cattle with Prolactin and Kappa casein polymorphism.

Genotype	Wild	Heterozygote	Mutation
Prolactin P	21	43	56
Kappa casein P	74	38	8

4.2. Prolactin and kappa casein genes polymorphism:

4.2.1. milk analysis of cross-breed dairy cattle with prolactin and kappa casein genes polymorphism.

The mean percentage protein % in the milk of cross-breed cows was significant ($P \leq 0.05$) with prolactin gene polymorphism. It found an increase in the AA genotype recorded as 4.31, compared with a decrease in AG and GG genotype was recorded as 3.56 and 3.49 for AG heterozygote, and GG genotype, respectively Table (4-2). on the other hand, the mean percentage protein % it was a significant increase ($P \leq 0.05$) with kappa casein gene polymorphism in AA genotype wild and it is recorded as 3.72 compared with a decrease in two other genotype AB

heterozygote, BB polymorphism has recorded a 3.132 and 3.183, respectively, Table (4-3).

The mean percentage (lactose %) was a significant increase (P≤ 0.05) with prolactin gene polymorphism as 6.27 in the AA wild genotype and a decrease to 5.05 for GG polymorphism and 5.1 for AG heterozygote genotype, respectively, Table (4-2). and the mean percentage (lactose %) was non-significant difference with kappa casein gene polymorphism it found an increase in 5.24, 5.02 compare with decrease in 4.94 for AA wild, AB heterozygote and BB mutation, respectively Table (4-3).

The mean freezing point in the milk of cross-breed cows was non-significant differences in the freezing point with prolactin gene polymorphism. It found non-significant differences in the levels of three genotype AA wild, AG heterozygote, GG genotype for 59.21, 60.11 and 59.86 respectively, Table (4-2), as well as, the mean freezing point c among Kappa casein gene polymorphism it was significant decrease (P≤ 0.05) in the AA genotype. It was recorded as 51.512 and different with two other genotype. It was an increase in AB heterozygote, and a decrease in GG polymorphism. It was recorded as 54.068, 51.512 respectively, Table (4-3).

The mean of density g/cm3 in the milk of cross-breed cows was non-significant difference with prolactin gene polymorphism. It was found as 30.32, 30.47 and 30.71 for AA wild, AG heterozygote, GG polymorphism, respectively, Table (4-2), as well as, The mean density g/cm3 was significant (P≤ 0.05) difference with kappa casein gene polymorphism. It found a decrease in BB polymorphism as 24.150 and AB heterozygote as 26.112 compared with an increase in AA genotype as 27.265, Table (4-3).

The mean percentage of (Fat %) in the milk of cross-breed cows was significant (P≤ 0.05) differences in the fat % with prolactin gene polymorphism. It was an increase in the AG heterozygote as 5.32 compared with a decrease in GG polymorphism and AA wild for 4.16 and 4.87, respectively, Table (4-2), the mean percentage (Fat %) was significant (P≤ 0.05) difference with kappa casein gene polymorphism. It was a decrease in the BB polymorphism as 4.33 compared with an increase in other two genotype. They are recorded as 5.003 and 5.003 for AA, AB heterozygote, respectively Table (4-3).

The mean percentage Milk solid non fat (MSNF%) in the milk with cow cross-breed with prolactin gene polymorphism among three genotype was non-significant difference. It found 9.25, 9.39 and 9.23 for AA wild, AG heterozygote, GG mutation, respectively Table (4-2), as well as, the mean percentage of Milk solid non fat % in the milk of cross-breed cows was significant (P≤ 0.05) differences in the kappa casein gene polymorphism. It found a decrease in GG polymorphism as 7.803 compared with an increase two other genotype AB heterozygote, AA wild. They are recorded as 8.210 and 8.296, respectively, Table (4-3).

Mean percentage of Czen in the milk of cross-breed cows was non-significant differences in the Czen with kappa casein gene polymorphism. It was recorded as 2.546, 2.506 and 2.380 for three genotype AA wild, AB heterozygote, BB polymorphism, respectively Table (4-3).

Table 4-2 : Mean and standard deviation of milk protein %, lactose %, freezing point c, density g/cm3, fat % and solid non fat % in the milk of cow cross-breed with prolactin gene polymorphism among three genotype. Prolactin genotype AA: Homozygote wild genotype, AG: Heterozygote genotype, GG: Homozygous.

Genotype of Prolactin	Protein %	lactose %	freezing point c	density g/cm3	fat %	solid non fat %
AA	4.31 ±0.93 A	6.27 ±1.45 A	59.86 ±11.45 A	30.71 ±7.15 A	4.87 ±1.16 A	9.25 ±0.68 A
GA	3.56 ± 0.25 B	5.1 ± 0.4 B	60.11 ± 4.78 A	30.47 ± 4.22 A	5.32 ± 1.4 B	9.39 ± 0.67 A
GG	3.49 ±0.65 B	5.05 ±0.99 B	59.21 ±4.43 A	30.32 ±3.28 A	4.16 ±0.85 A	9.23 ±1.79 A

Table 4-2 different letter represented significant same letter represented non-significant

Table 4-3 : Mean and standard deviation of milk protein %, lactose %, freezing point c, density g/cm3, fat %, solid non fat % and Czen % in the milk of cow cross-breed with kappa casein gene polymorphism among three genotype. Kappa casein genotype AA: Homozygote wild genotype, AB: Heterozygote genotype, BB: Homozygous.

Genotype of K casein	protein %	lactose %	freezing point c	density g/cm3	fat %	solid non fat %	Czen %
AA	3.72 ± 0.078 A	5.24 ± 1.45 A	51.512 ± 2.361 A	27.265 ± 7.21 A	5.003 ± 0.312 A	8.296 ± 0.159 A	2.546 ± 0.054 A
AB	3.132 ± 0.028 B	5.02 ± 0.4 A	54.068 ± 0.678 B	26.112 ± 0.965 B	5.003 ± 0.312 A	8.21 ± 0.117 A	2.506 ± 0.022 A
BB	3.183 ± 0.068 B	4.94 ± 0.99 B	26.01 ± 5.11 C	24.15 ± 0.965 C	4.33 ± 0.446 B	7.803 ± 0.217 B	2.38 ± 0.062 A

Table 4-3 different letter represented significant same letter represented non-significant

4.2.2. Trace elements analysis of cross-breed dairy cattle in prolactin and kappa casein genes polymorphism.

The mean serum concentration of Serum Iron µg/dL in the blood serum of cross-breed cows was significant (P≤ 0.05) differences in the Serum of cattle with prolactin gene polymorphism. It was an increase recorded in the AA genotype as 126.86 and it has more concentration compared with a decrease in AG heterozygote and GG polymorphism. The serum concentration was recorded as 109.45 and 116.59 respectively Table (4-4), while, the mean concentration of Serum Iron µg/dL in the blood serum of cross-breed cows was significant (P≤ 0.05) differences in the Serum Iron µg/dL with kappa casein gene polymorphism. It found an increase in AA genotype recorded as 135.47 compared with a decrease in other two genotype. They are recorded as 121.32 and 113.64 for AB heterozygote, BB mutation, respectively, Table (4-5).

The mean of Serum Phosphorus mg/dL in the blood serum of cross-breed cows was non-significant difference with prolactin gene polymorphism. It was found as 5.82, 5.21 and 5.78, for AA wild, AG heterozygote, GG polymorphism respectively Table (4-4), Otherwise, the mean Serum Phosphorus mg/dL was a significant (P≤ 0.05) difference with kappa casein gene polymorphism. It found a decrease in BB polymorphism as 4.01 compared with other two genotype AA wild, AB heterozygote recorded as 5.42 and 5.34 for respectively Table (4-5).

Generally, the mean concentration Serum of Calcium mg/dL in the blood serum of cross-breed cows was non-significant difference. It found 8.52, 8.72 and 8.39 for AA wild, AG heterozygote, GG polymorphism respectively Table (4-4), The mean concentration Serum Calcium mg/dL was non-significant difference with kappa casein gene polymorphism. It

was found as 8.31, 8.54 and 8.11 for AA wild, AB heterozygote, BB polymorphism, respectively Table (4-5).

Table 4-4 : mean and standard deviation of Serum Iron µg/dL, Serum Phosphorus mg/dL and Serum Calcium mg/dL in the milk with cow cross-breed with prolactin gene polymorphism among three genotype. Prolactin genotype AA: Homozygote wild genotype, AG: Heterozygote genotype, GG: Homozygous mutation.

Genotype of prolactin	Serum Iron µg/dL	Serum Phosphorus mg/dL	Serum Calcium mg/dL
AA	126.86 ± 8.24 A	5.82 ± 1.23 A	8.52 ± 0.68 A
AG	109.45 ± 29.93 B	5.21 ± 1.13 A	8.72 ± 0.73 A
GG	116.59 ± 33.45 C	5.78 ± 1.25 A	8.39 ± 0.54 A

Table 4-4 different letter represented significant same letter represented non-significant

Table 4-5 : mean and standard deviation of Serum Iron µg/dL, Serum Phosphorus mg/dL and Serum Calcium mg/dL in the milk with cow cross-breed with kappa casein gene polymorphism among three genotype. Kappa casein genotype AA: Homozygote wild genotype, AB: Heterozygote genotype, BB: Homozygous mutation.

Genotype of K casein	Serum Iron µg/dL	Serum Phosphorus mg/dL	Serum Calcium mg/dL
AA	135.47 ± 3.21 A	5.42 ± 1.03 A	8.31 ± 0.11 A
AB	121.32 ± 11.41 B	5.34 ± 1.12 A	8.54 ± 0.29 A
BB	113.64 ± 9.31 C	4.01 ± 0.99 B	8.11 ± 0.88 A

Table 4-5 different letter represented significant same letter represented non-significant

4.2.3. Hormonal analysis of cross-breed dairy cattle with prolactin and kappa casein genes polymorphism.

On the other hand , the mean concentration of Prolactin Hormone (PRL) mIU/L in the blood serum of cross-breed among Prolactin gene polymorphism was significant (P≤ 0.05) difference. They found a decrease in GG polymorphism as 91.77 and in AG genotype as, 119.69 compared with an increase of 127.7 for AA wild, respectively Table (4-6), as well as, the mean of serum concentration Prolactin Hormone (Prl) mIU/L in the blood serum of cross-breed was significant (P≤ 0.05) difference with kappa casein gene polymorphism. It increased in the AA wild as 124.4 compared with a decrease in other two genotype AB heterozygote, BB mutation as 118.66 and 120.98 respectively Table (4-7).

The mean serum concentration of Growth Hormone (GH) µIU/mL in the blood serum of cross-breed cows was non-significant differences in the Growth hormone µIU/mL with prolactin gene polymorphism. It was found as 2.84, 2.61 and 2.38 for AA wild, AG heterozygote, GG polymorphism, respectively Table (4-6), Otherwise, the Mean of serum concentration of Growth Hormone (GH) µIU/mL was non-significant differences in the growth hormone µIU/mL with kappa casein gene polymorphism. It found 2.91, 2.73 and 2.49 for AA wild, AB heterozygote, BB polymorphism, respectively, Table (4-7).

The mean serum concentration of Thyroid Stimulating Hormone (TSH) mIU/L in the blood serum of cross-breed cows was significant (p≤ 0.05) difference with prolactin gene polymorphism. It found increases as 25.21 for AA wild compared with AG heterozygote 23.94, and a decrease in the GG genotype 22.48, Table (4-6), at the same time, the mean serum concentration Thyroid Stimulating Hormone (TSH) mIU/L was

significant (P≤ 0.05) difference with kappa casein gene polymorphism. It found an increase in AA wild as 24.87 compared with a decrease in the other two genotype AB heterozygote recorded as 22.98 and BB polymorphism recorded as 21.15 for respectively Table (4-7).

The mean serum concentration of Progesterone Hormone (Pro) ng/mL in the blood serum of cross-breed cows was significant (P≤ 0.05) differences in the Progesterone ng/mL with prolactin gene polymorphism. It found an increase in AA wild as 7.52 compared with two genotype AG heterozygote 7.93 and decrease in the GG polymorphism. It was recorded as 4.51, Table (4-6), on the other hand, the mean serum concentration of Progesterone Hormone (Pro) ng/mL in the blood serum of cross-breed cows was non-significant differences in the Progesterone ng/mL with kappa casein gene polymorphism. It found 7.81, 7.51 and 7.73 for AA wild, AB heterozygote, BB polymorphism respectively, Table (4-7).

Otherwise , the mean serum concentration of Estrogen (Estradiol) (Es) pg/mL in the blood serum of cross-breed cows was non-significant difference with prolactin gene polymorphism. It found10.15, 10.76 and 10.01 for AA wild, AG heterozygote, GG polymorphism, respectively Table (4-6), as well as, The mean serum concentration Estrogen (Estradiol) Hormone pg/mL in the blood serum of cross-breed cows was non-significant difference with kaapa casein gene polymorphism. It was account 10.43, 10.66 and 10.09 for AA wild, AB heterozygote, BB polymorphism respectively Table (4-7).

On the other hand , the mean serum concentration of Oxytocin Hormone (OXY) mIU/L in the blood serum of cross-breed cows was non-significant difference with prolactin gene polymorphism. It found an

increase in AA wild for 18.15 compared with 17.68 and 17.3 for AG heterozygote, GG mutation, respectively Table (4-6), as well as, The mean serum concentration Oxytocin Hormone (OXY) mIU/L in the blood serum of cross-breed cows was significant ($P \leq 0.05$) difference with kappa casein gene polymorphism. It found an increase in AA wild recorded as 18.82 compared with a decrease in two genotype AB heterozygote, BB polymorphism recorded as 17.93 and 17.74 for respectively Table (4-7).

Table 4-6 : mean and standard deviation of Prolactin Hormone (Prl) mIU/L, Growth Hormone µIU/mL, Thyroid Stimulating Hormone mIU/L, Progesterone ng/mL, Estrogen (Estradiol) pg/mL and Oxytocin in the milk with cow cross-breed with prolactin gene polymorphism among three genotype. Prolactin genotype AA: Homozygote wild genotype, AG: Heterozygote genotype, GG: Homozygous mutation.

Genotype of prolactin	Prolactin mIU/L	Growth hormone µIU/mL	Thyroid Stimulating Hormone mIU/L	Progesterone ng/mL	Estrogen (Estradiol) pg/mL	Oxytocin
AA	127.7 ± 33.42 A	2.84 ± 0.72 A	25.21 ± 5.24 A	7.52 ± 1.99 A	10.15 ± 0.533 A	18.15 ± 3.31 A
AG	119.69 ± 32.99 B	2.61 ± 0.76 A	23.94 ± 3.54 B	7.93 ± 2.11 A	10.76 ± 0.94 A	17.68 ± 3.58 A
GG	91.77 ± 18.99 C	2.38 ± 0.41 A	22.48 ± 3.62 C	4.51 ± 1.42 B	10.01 ± 0.523 A	17.3 ± 3.69 A

Table 4-6 different letter represented significant same letter represented non-significant

Table 4-7 : mean and standard deviation of Prolactin Hormone (Prl) mIU/L, Growth Hormone µIU/mL, Thyroid Stimulating Hormone mIU/L, Progesterone ng/mL, Estrogen (Estradiol) pg/mL and Oxytocin in the milk with cow cross-breed with kappa casein gene polymorphism among three genotype. Kappa casein genotype AA: Homozygote wild genotype, AB: Heterozygote genotype, BB: Homozygous mutation.

Genotype of k casein	Prolactin mIU/L	Growth hormone µIU/mL	Thyroid Stimulating Hormone (TSH)	Progesterone ng/mL	Estrogen (Estradiol) pg/mL	Oxytocin
AA	124.4 ±11.42 A	2.91 ±0.78 A	24.87 ±6.34 A	7.81 ±1.98 A	10.43 ±0.43 A	18.82 ±4.31 A
AB	118.66 ±25.42 B	2.73 ±0.74 A	22.98 ±3.45 B	7.51 ±2.31 A	10.66 ±1.42 A	17.93 ±2.63 B
BB	120.98 ±22.55 C	2.49 ±0.72 A	21.15 ±2.33 C	7.73 ±2.02 A	10.09 ±0.78 A	17.74 ±3.42 B

Table 4-7 different letter represented significant same letter represented non-significant

4.2.4. Hematological analysis of cross-breed dairy cattle with prolactin and kappa casein genes polymorphism.

Mean level WBC x 10^9/L in the blood parameters of cross-breed cows was significant ($P \leq 0.05$) differences in the WBC with prolactin gene polymorphism. It was an increase in AA wild recorded as 36.18 compared with a decrease in two genotype AG heterozygote, GG polymorphism. It was recorded as 29.18 and 27.3 respectively Table (4-8), Otherwise, Mean level WBC x 10^9/L in the blood parameters of cross-breed cows was significant ($P \leq 0.05$) differences in the WBC with kappa casein gene polymorphism. It found an increase in AA wild recorded as 35.14 compared with a decrease in two genotype AB heterozygote, BB polymorphism recorded as 28.49 and 25.11 for respectively, Table (4-9).

The mean level RBC x 10^{12}/L in the blood parameters of cross-breed cows was non-significant difference with prolactin gene polymorphism it was found 3.35, 2.91 and 3.05 for AA wild, AG heterozygote, GG polymorphism, respectively Table (4-8), on the other hand, the mean level RBC x 10^{12}/L in the blood parameters of cross-breed cows was non-significant difference with kappa casein gene polymorphism. It was found 3.18, 3.02 and 3.14 for AA wild, AB heterozygote, BB polymorphism, respectively, Table (4-9).

The mean of Hb g/dL in the blood parameters of cross-breed cows was non-significant difference with prolactin gene polymorphism as 10.91, 9.45 and 10.05 for AA wild, AG heterozygote, GG polymorphism, respectively Table (4-8), on the other hand, the mean Hb g/dL in the blood parameters of cross-breed cows was non-significant difference with kappa casein gene polymorphism. It found 10.21, 10.34 and 10.19 for AA wild, AB heterozygote, BB polymorphism, respectively Table (4-9).

Mean percentage PCV % in the blood parameters of cross-breed cows was significant (P≤ 0.05) differences in the (PCV %) with Prolactin gene polymorphism. It was an increase in AA wild it was recorded as 16.01 compared with a decrease in two genotype AG heterozygote, GG polymorphism recorded as 13.94 and 14.68 respectively Table (4-8), Otherwise, the mean percentage (PCV %) in the blood parameters of cross-bred cows was non-significant differences in the HCT with kappa casein gene polymorphism. It found 15.32, 14.09 and 15.23 for AA wild, AB heterozygote, BB polymorphism, respectively, Table (4-9).

On the other hand, The mean MCV fL in the blood parameters of cross-bred cows was non-significant difference with prolactin gene polymorphism. It found 48.16, 48.1 and 47.94, for AA wild, AG heterozygote, GG polymorphism, respectively Table (4-8), as well as, the mean MCV fL in the blood parameters of cross-bred cows was non-significant difference with kappa casein gene polymorphism. It found 48.53, 48.04 and 48.13 for AA wild, AB heterozygote, BB polymorphism, respectively Table (4-9).

Otherwise, The mean concentration MCH pg in the blood parameters of cross-bred cows was non-significant difference with prolactin gene polymorphism. It found 32.96, 32.73 and 32.65, for AA wild, AG heterozygote, GG mutation, respectively Table (4-8), on the other hand, the mean MCH pg in the blood parameters of cross-bred cows was non-significant difference with kappa casein gene polymorphism. It found 32.09, 32.21 and 31.94 for AA wild, AB heterozygote, BB polymorphism, respectively Table (4-9).

The mean MCHC g/dL in the blood parameters of cross-bred cows was non-significant difference with prolactin gene polymorphism. It

found 69.2, 68.74, 68.56 for AA wild, AG heterozygote, GG mutation, respectively Table (4-8), as well as the mean MCHC g/dL in the blood parameters of cross-bred cows was non-significant difference with kappa casein gene polymorphism. It found 68.44, 68.13 and 68.04, for AA wild, AB heterozygote, BB polymorphism, respectively, Table (4-9).

Table 4-8 : mean and standard deviation of WBC x 10^9/L, RBC x 10^{12}/L, HGB g/dL, HCT (PCV) %, MCV /fL, MCH pg and MCHC g/dL in the milk with cow cross-breed with Prolactin gene polymorphism among three genotype. Prolactin genotype AA: Homozygote wild genotype, AG: Heterozygote genotype, GG: Homozygous mutation.

Genotype of prolactin	WBC	RBC	HGB	HCT	MCV	MCH	MCHC
AA	36.18 ±7.08 A	3.35 ±0.53 A	10.91 ±2.71 A	16.01 ±2.71 A	48.16 ±3.92 A	32.96 ±2.93 A	69.2 ±9.21 A
AG	29.18 ±5.29 B	2.91 ±0.64 A	9.45 ±1.82 A	13.94 ±3.15 B	48.1 ±4.43 A	32.73 ±2.87 A	68.74 ±7.47 A
GG	27.3 ±6.8 C	3.05 ±0.49 A	10.05 ±1.62 A	14.68 ±2.65 C	47.94 ±3.75 A	32.65 ±2.98 A	68.56 ±6.22 A

Table 4-8 different letter represented significant same letter represented non-significant

Table 4-9 : mean and standard deviation of WBC x 10^9/L, RBC x 10^{12}/L, HGB g/dL, HCT (PCV) %, MCV /fL, MCH pg and MCHC g/dL in the milk with cow cross-breed with Kappa casein gene polymorphism among three genotype. Kappa casein genotype AA: Homozygote wild genotype, AB: Heterozygote genotype, BB: Homozygous mutation.

Genotype Of k casein	WBC	RBC	HGB	HCT	MCV	MCH	MCHC
AA	35.14 ±4.41 A	3.18 ±0.78 A	10.21 ±1.93 A	15.32 ±2.42 A	48.53 ±1.41 A	32.09 ±1.36 A	68.44 ±6.63 A
AB	28.49 ±8.33 B	3.02 ±0.84 A	10.34 ±1.16 A	14.09 ±1.64 A	48.04 ±1.44 A	32.21 ±1.61 A	68.13 ±6.63 A
BB	25.11 ±3.41 C	3.14 ±0.89 A	10.19 ±1.54 A	15.23 ±2.04 A	48.13 ±1.11 A	31.94 ±1.15 A	68.04 ±5.11 A

Table 4-9 different letter represented significant same letter represented non-significant

4.3.1.Correlation between Prolactin gene polymorphism with serum production hormones concentration

Table (4.10) represents the mean concentration of (GH, TSH, PRL, OXY, PRO and ES) hormones in serum of dairy cattle for three (Prolactin) genotypes (AA, AG & GG) groups of dairy cattle breeds in Karbala province.

The result found a positive strong correlation (0.834) between thyroid stimulating hormone (TSH) with Progesterone hormones(PRO) among cattle have AA genotype, and mid correlation (0.527) between growth hormone and Estrogen in cattle have AA genotypes.

Table (4.10):correlation Homozygous genotype (AA) group with six hormones (GH, PRL, TSH, OXY, PRO and ES) of dairy cattle breeds in Karbala province

AA	Growth hormone µIU/mL	Thyroid Stimulating Hormone (TSH)	Prolactin	Progesterone ng/mL	Estrogen (Estradiol) pg/mL	Oxytocin
Growth hormone µIU/mL	1					
Thyroid Stimulating Hormone (TSH)	0.03	1				
Prolactin	-0.33	0.24	1			
Progesterone ng/mL	0.30	0.83 **	-0.26	1		
Estrogen (Estradiol) pg/mL	0.52 *	-0.11	-0.21	-0.32	1	
Oxytocin	-0.02	0.08	-0.20	0.36	-0.26	1

Table (4-10) * was significant (0.05) and ** was significant (0.01)

Table (4.10) represents the mean concentration of (GH, PRL, TSH, OXY, PRO and ES) hormones in serum of dairy cattle for three (Prolactin) genotypes (AA, AG & GG) groups of dairy cattle breeds in Karbala province.

The result did not find any correlation between milk production hormones with Prolactin gene polymorphism AG genotype

Table (4.11):correlation heterozygous genotype (AG) group with six hormones (GH, PRL, TSH, OXY, PRO and ES) of dairy cattle breeds in Karbala province

AG	Growth hormone µIU/mL	Thyroid Stimulating Hormone (TSH)	Prolactin	Progesterone ng/mL	Estrogen (Estradiol) pg/mL	Oxytocin
Growth hormone µIU/mL	1					
Thyroid Stimulating Hormone (TSH)	0.35	1				
Prolactin	0.03	-0.02	1			
Progesterone ng/mL	-0.12	-0.12	-0.11	1		
Estrogen (Estradiol) pg/mL	0.19	0.07	0.00	0.24	1	
Oxytocin	0.19	0.23	-0.14	0.11	0.19	1

Table (4.11) represents the mean concentration of (GH, PRL, TSH, OXY, PRO and ES) hormones in serum of dairy cattle for three (Prolactin) genotypes (AA, AG & GG) groups of dairy cattle breeds in Karbala province.

The result found a negative mid correlation between prolactin milk production hormones with Progesterone in the cattle have Prolactin gene polymorphism GG genotype**.**

Table (4.12):correlation Homozygous Mutant (GG) group with six hormones (GH, PRL, TSH, OXY, PRO and ES) of dairy cattle breeds in Karbala province

GG	Growth hormone µIU/mL	Thyroid Stimulating Hormone (TSH)	Prolactin	Progesterone ng/mL	Estrogen (Estradiol) pg/mL	Oxytocin
Growth hormone µIU/mL	1					
Thyroid Stimulating Hormone (TSH)	-0.12	1				
Prolactin	0.105	0.33	1			
Progesterone ng/mL	-0.09	0.11	-0.57	1		
Estrogen (Estradiol) pg/mL	0.24	-0.17	0.07	0.03	1	
Oxytocin	-0.09	0.37	0.05	-0.13	-0.15	1

4.3.2.Analysis of milk production of cross-breed dairy cows with prolactin gene polymorphism.

Table (4.13) Mean of milk quantity of prolactin gene among three genotypes (AA,GG&AG) groups for three months of dairy cattle breeds in Karbala province.

Milk quantity/month / Genotype	Mean of 1 month kg/day Mean ± SD	Mean of 2 month kg/day Mean ± SD	Mean of 3 month kg/day Mean ± SD
AA	19.94±0.78 A	21.83±1.45 A	20.11±0.67 A
AG	15.10±0.92 B	16.22±2.12 B	14.8±2.34 B
GG	13.42±1.62 C	14.36±1.33 C	13.82±0,98 C

Table (4.13) different letter represented significant same letter represented non significant.vertical

The table (4.13) showed a significant difference ($p<0.05$) in milk quantity between three prolactin genotypes (AA, AG and GG) groups for three months. The genotype (GG) group result showed a significant decrease compared with other genotype ($p<0.05$) in milk quantity. It was recorded as (19.94, 15.10 and 13.42) for three genotupe in first month respectively and it found an increase in AA group compared with a decrease in AG an GG groups

On other hand, our results revealed the genotype (AA) group increased significantly in milk production ($p<0.05$) in comparison with (AG) group and (GG) Group second month, as well as it was noticed that they increased significantly ($p<0.05$) in (20.11) for (AA) compare with AG and GG decreased in milk production in third month.

4.4.1. Receiver operating characteristic for prediction of Prolactin levels in cattle.

ROC analysis demonstrated an AUC of 0.789. a Prolactin drop of 80 % was the point of optimal discrimination with a sensitivity of 65% and specificity of 92% for predicting A allele. The prediction value for A allele was recorded as > 95.63 mIU/L, figure 4-1.

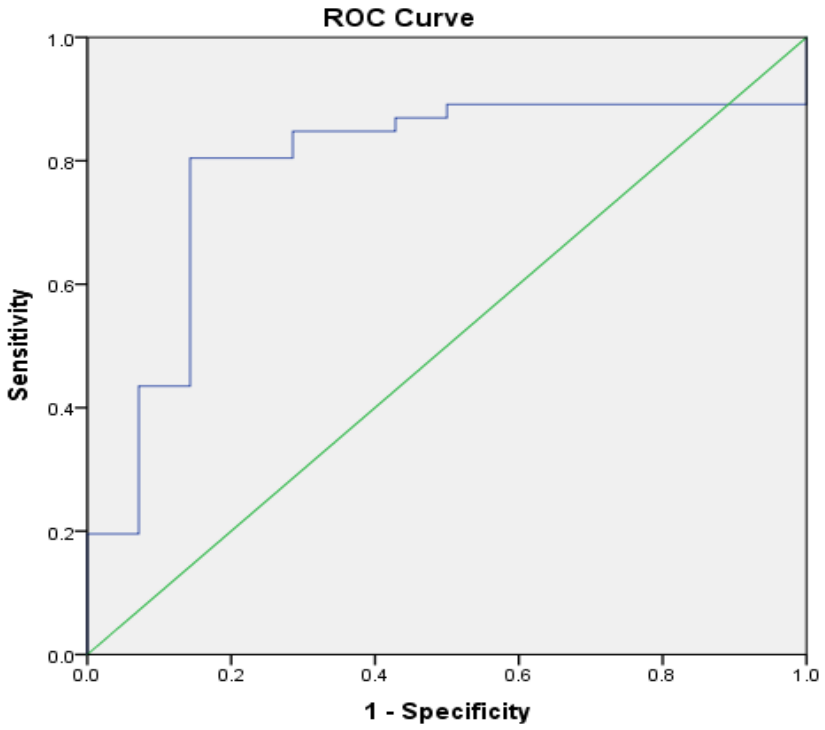

Figure (4-1). ROC analysis for prediction of Prolactin levels in cattle

Table (4.14) Area Under the Curve for prediction of Prolactin levels in cattle

Test Result Variable(s): Prolactin levels in cross bred cattle

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.789	.069	.001	.653	.925

4.4.2.Receiver operating characteristic for prediction of kappa casein levels in cattle.

ROC analysis demonstrated an AUC of 0.929. a kappa casein drop of 92 % was the point of optimal discrimination with a sensitivity of 82% and specificity of 100% for predicting A allele. The prediction value for A allele was recorded as > 3.51

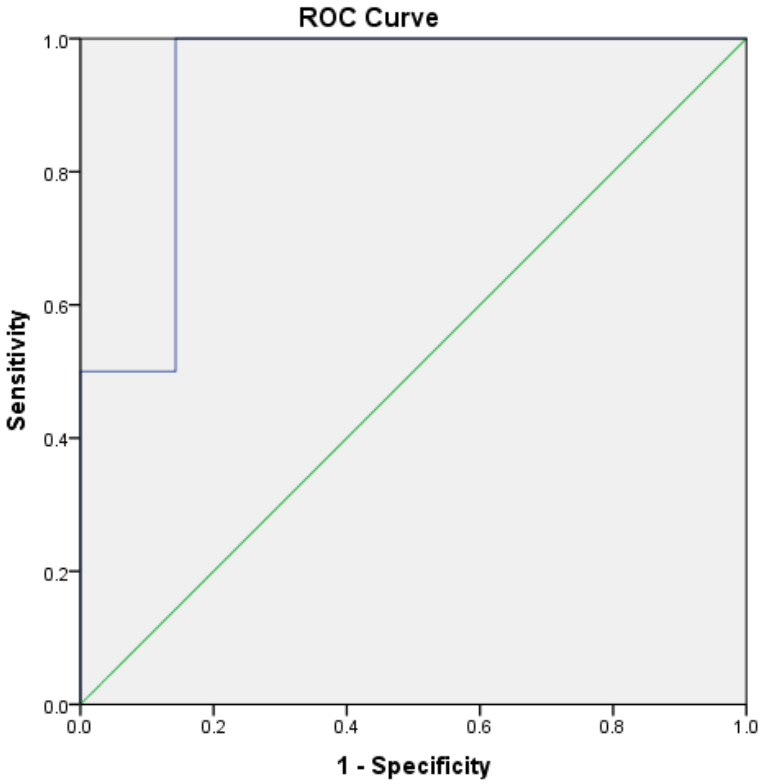

Figure (4-2). ROC analysis for prediction of Prolactin levels in cattle

Table (4.15) Area Under the Curve for prediction of kappa casein levels in cattle .

Test Result Variable(s): kappa casein levels in cross bred cattle

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.929	.052	.000	.827	1.000

a. Under the nonparametric assumption

4.4.3.Analysis of milk composition of cross-breed dairy cows with kappa casein gene polymorphism.

Table (4.16) represented mean percentage of (lactose %, freezing point c, protein %, density g/cm3, solid non fat %, fat %) milk composition of dairy cattle for three (kappa casein) genotypes (AA, AB & BB) groups of dairy cattle breeds in Karbala province.

Our results found insignificant for all milk component , except for protein and fat and freezing point, the percentage of protein was high in cows carrying the AA allele 3.72% , which is higher than the percentage of protein in cows carrying the allele AB and BB alleles. It was found as 3.132% and 3.183% respectively.

Table (4.16) showed a significant increase ($p<0.05$) in the fat percentage at genotype (AA) group as (5.003%) compared with BB groups , while the fat percentage shows a significant a decrease ($p\leq0.05$) in (BB) group (4.33) compared with (AB) and (AA) groups, as well as, in same table showed a significant increase ($p\leq0.05$) in freezing point c at genotype AA group as 51.512 compared with BB groups it was recorded as 26.01.

Table (4.16) Analysis of milk composition with kappa casein gene polymorphism

Genotype of K casein	lactose %	freezing point c	protein %	density g/cm3	solid non fat %	fat %
AA	5.24 ±1.45 A	51.512 ±2.361 A	3.72 ±0.078 A	27.265 ±7.21 A	8.296 ±0.159 A	5.003 ±0.312 A
AB	5.02 ±0.4 A	54.068 ±0.678 B	3.132 ±0.028 B	26.112 ±0.965 B	8.21 ±0.117 A	5.003 ±0.312 A
BB	4.94 ±0.99 A	26.01 ±1.22 C	3.183 ±0.068 B	24.15 ±0.965 C	7.803 ±0.217 B	4.33 ±0.446 B

Table (4.16) different letter represented significant same letter represented non significant

4.5.1. PCR amplification of milk Prolactin gene.

High quality genomic DNA was used as a template for the amplification of milk PRL gene, The amplicons of the this gene (PRL) were presented (Figure 4-3) and sequence results clearly indicate that the correct target genes were investigated in this study.

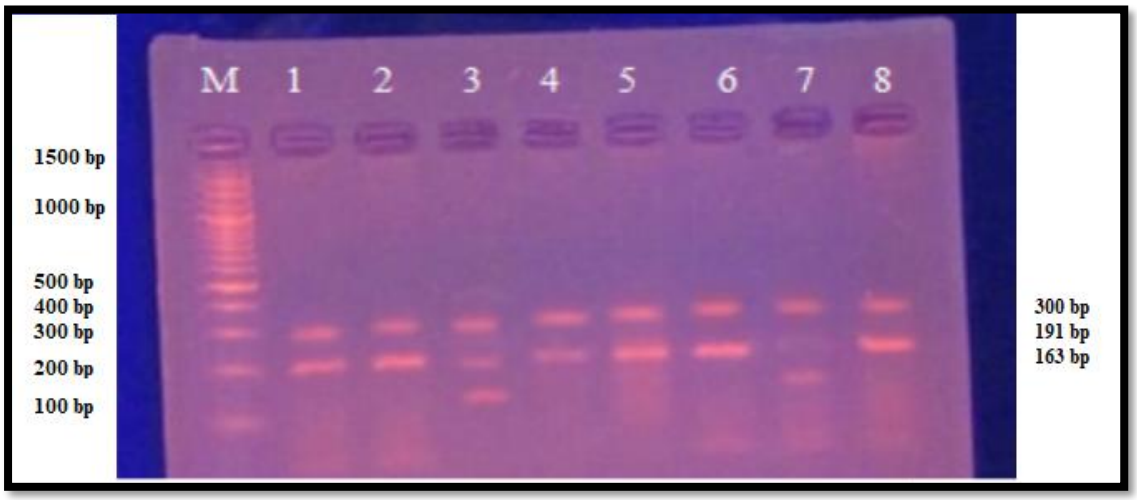

Figure (4-3) The genotypic analysis of PRL gene in cross bread Iraqi cattle. PCR products amplified from genomic DNA collected from blood samples extracted from Karbala city cattle. The homozygous (GG) genotypes appeared a two bands (163 bp and 300 bp) which represented as 7 wells, The homozygous (AA) genotypes appeared a two bands (191 bp and 300 bp) which represented as 1,2,4,5,6 and 8 wells and heterozygous (AG) genotype appeared a three bands (191 bp , 163 bp and 300 bp), which represented 3 well.

4.5.2.PCR amplification of milk Kappa casein gene.

High quality genomic DNA was used as a template for the amplification of milk K-casein gene, The amplicons of the this gene (K-casein) were presented (Figure 4-4) and sequence results clearly indicate that the correct target genes were investigated in this study.

The PCR product of the kappa-casein gene using specific set of primers (CSN3-F and CSN3-R) was a fragment of 453 bp DNA. Digestion of 453 bp fragment of kappa-casein gene by HinfI restriction endonuclease generated four fragments, i.e. 453, 326, 100 and 27 bp (Fig 4-4). Three fragments of 326, 100 and 27 bp represent homozygotes A allele were in four fragments viz. 453, 326, 100 and 27 bp represent heterozygotes AB for kappa-casein gene. BB genotypes were observed among the studied population no digested bands .

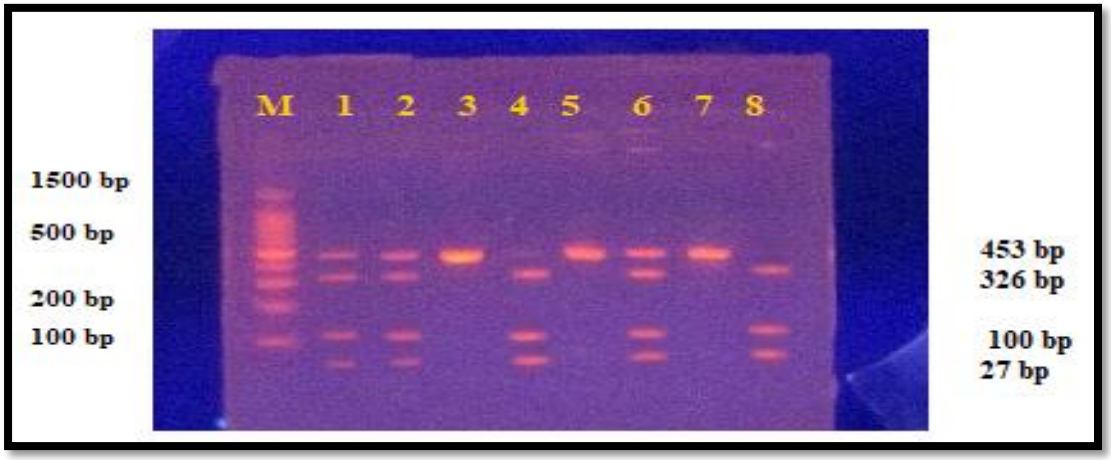

Figure (4-4): PCR-RFLP product of kappa-casein gene. After the PCR product was digested by HinfI and visualized on 1% agarose gel, the results were the 453 bp fragment of uncut PCR product representing homozygotes B allele (3 , 5 and 7 wells), three fragments of 326, 100 and 27 bp representing homozygotes A allele(4, and 8 well) , and four fragments 453, 326, 100 and 27 bp representing heterozygotes (A/B) for kappa-casein gene (1,2 and 6 wells).

Chapter Five

Discussion

5.Discussion:

5.1. Prolactin and kappa casein genes polymorphism:

5.1.1. milk analysis of cross-breed dairy cattle with prolactin and kappa casein genes polymorphism.

Milk protein yield is of great significance for the dairy industry, the amount and composition of proteins in milk is largely determined by the genetics of the animal, and is difficult to change through nutrition (Heck *et al*., 2009).

However, due to the high requirement of protein synthesis for energy, the milk protein yield can be affected by the energy content in the diet, physiological status of animal and genetic factors (Dai *et al*., 2017).

Our result found out that all milk composition had a significant increase in AA genotype and a decrease in GG genotype within prolactin genotype and also it found an increase in AA genotype compared with a decrease in BB genotype within kappa casein genotype, otherwise, protein and lactose were given a significant associated between milk composition and genotype. The mean percentage protein % in the milk of cross-breed cows was significant ($P \leq 0.05$) with prolactin gene polymorphism. It found an increase in the AA genotype, compared with a decrease in AG and GG genotypes respectively, Table (4-2).

This result was in agreement with (Lacasse *et al*., 2016) who found that PRL injections were not sufficient to restore milk yield, they tended to increase milk protein and lactose yields and increased the viability of mammary epithelial cells purified from milk, the current study found the G allele was unfavorable for milk and protein yield, this result was also in

accordance with (Mehmannavaz *et al*., 2009) who worked on cattle breed in Iran.

Preliminary analysis showed some significant differences associations between PRL genotypes and milk performance traits, although local breed GG polymorphism, cows yielded more milk with higher protein content in the first lactation. This results was in disagreement with (Brym *et al*., 2005) who was found there was no significant associations between PRL genotypes and milk performance traits, he found that Black-and-White cows with genotype AG showed the highest milk yield, while cows with genotype GG showed the highest fat content.

The causes for the apparent difference in protein concentration and its relationship with the Prolactin hormones was instability of the measurement of the concentration of milk hormone in the blood of cows, some of studies were noted that the concentration of prolactin (PRL) in whole milk of cows is substantially higher during the few days before parturition than after parturition (Auchtung *et al*., 2005). The concentrations of Prolactin in milk decreased abruptly after parturition and rapidly approached concentrations found in blood plasma, the induction of prepartum lactogenesis by regular prepartum milking in previous studies did not decrease the elevated concentration of PRL in prepartum mammary secretions (Gross *et al*., 2014).

This result can be investigated through a positive express of AG heterozygote, allele on the milk and protein yield , otherwise it was less extended on fat yield Fat percentage was lower because of the higher milk yield ,but nearly constant fat yield, associated with the AG heterozygote, allele (Boleckova *et al*., 2012) ,in addition the effect of prolactin hormone on milk components was clear ,because the prolactin

regulates several secreted milk proteins ,including the caseins ,lactoglobulin ,lactalbumin and whey acidic protein (Pegolo *et al*. ,2018).

On the other hand, The protein-containing major portions of caseins are relatively large aggregates of four molecules: αs1-casein (αs1-CN), αs2-casein (αs2-CN), β-casein (β-CN), and k-casein (k-CN) (3). After casein, other parts of proteins are whey or serum proteins that are composed of α-lactalbumin (α-La), β-lactoglobulin (β-Lg), immunoglobulin (Ig), bovine serum albumin (BSA), proteoses, and peptones (Fox, 2003).

Our result found out that the percentage of Czen in the milk of cross-breed cows was non-significant differences in the Czen with kappa casein gene polymorphism, for three genotype AA wild, AB heterozygote, BB polymorphism, respectively table (4-3). While in the mean percentage protein % it was significant increase ($P \leq 0.05$) with kappa casein gene polymorphism in AA genotype wild compared with two other genotype AB heterozygote, BB polymorphism respectively, Table (4-3).

On the other hand, it found a significant increase of lactose concentration in cow milk composition and related with prolactin genotypes table (4-2), the concentration of prolactin had a highly significant decrease in the GG genotype rather than from AG heterozygote, and AA wild, genotypes table (4-2), as well as, the mean percentage (Fat %) was a significant ($P\leq 0.05$) difference with kappa casein gene polymorphism. It has a decrease in the BB polymorphism compared with other two genotype AA wild, AB heterozygote, respectively table (4-3).

Our result found that the mean percentage (lactose %) had a non-significant ($p \geq 0.05$) difference with kappa casein gene polymorphism it was found increase in 5.24, 5.02 compared with decrease in 4.94 for AA wild, AB heterozygote and BB mutation, respectively table (4-3). it was found an disagreement with Jawasreh and his colleagues was recorded that AA allele 5.02, and AB genotype was 4.90 and finally, BB was more significant association between milk production and composition with prolactin gene in awassi sheep (Jawasreh *et al*., 2019).

Lactose is the main carbohydrate in mammalian milk, and is responsible for the osmotic balance between the blood and the alveolar lumen of the breast. They are the main milk solids, and their composition and concentration in milk are mainly affected by the health of the udder, energy balance and metabolism of the cow (Zachut *et al*., 2020).

Our result found in the milk composition like lactose, fat %, solid non fat %, density g/cm3, freezing point c were non-significant within Kappa casein genotype, Table (4-3), they were recorded as otherwise, protein and Fat were given a significant associated with milk composition and genotype, Table (4-2) and (4-3).

This result can be illustrated by a positive express of B allele on the milk and protein yield , otherwise it was less extended on fat yield. The fat percentage was lower because of the higher milk yield ,but nearly constant fat yield, associated with the B allele (Cosier *et al*., 2007) ,in addition the effect of prolactin hormone on milk components was clear ,because the prolactin regulates several secreted milk proteins ,including the caseins ,lactoglobulin ,lactalbumin and whey acidic protein (Jameson & Grassman, 2016).

Gene expression experiments also proved the necessary effects of prolactin and its receptors on cow udder development. and the formation and expression of prolactin of milk protein gene (Jiang *et al*., 2012). So, bovine prolactin gene seems to be an excellent candidate for Quantity Trait loci (QTL) that affects milk production traits. Cattle Prolactin spans about 9.4 kb and consists of five exon and four introns, encoding mature protein Contains 199 amino acids (Dybus *et al*., 2005). First to paint it on cows chromosome.

PRL has proved mandatory role for mammary gland development, lactogenesis and expression of milk protein genes .Therefore, the bovine prolactin gene seems to be an excellent candidate for linkage analysis of quantitative trait loci (QTL) affecting milk production traits (Othman *et al*., 2011). The effects of Oxytocin on milk composition milk fatty acids and proteins are not clear yet. According to some scientists, oxytocin affects milk components in diverse ways, while some are of the view that milk composition is not affected (Hameed *et al*., 2016).

5.1.2. Tracing elements analysis of cross-breed dairy cattle in prolactin and kappa casein genes polymorphism.

The mean serum concentration of Iron in the blood of cross-breed cows had significant ($P \leq 0.05$) differences with prolactin gene polymorphism. It has an increase in the AA genotype and it has more concentration compared with AG heterozygote and GG polymorphism respectively, while, the mean concentration of Serum Iron of cross-breed cows has significant ($P \leq 0.05$) differences with kappa casein gene polymorphism. There was an increase in AA genotype compared with decrease in other two genotype AB heterozygote, BB mutation, respectively, Table (4-4) and (4-5).

Iron is an essential element for almost all living things, due to environmental and genetic factors, the iron concentration in tissues shows normal differences between individuals. Iron in the body, although it is a necessity, can also cause excessive toxicity through the production of reactive oxygen species (Soetan *et al*., 2010). Therefore, iron balance must be maintained according to the iron absorption rate, iron utilization rate, iron storage rate and rate systematically , Any gene that codes for a protein can be mutated Participating in maintaining iron balance has the ability to change iron load. Influences information (Duan, 2010).

On the other hand, Clusters of phosphoserine residues in cow milk caseins bind iron (Fe) with high affinity. Casein inhibits Fe absorption, but protein hydrolysis lessens this effect. Phosphopeptides from different caseins gave conflicting results on Fe absorption; release of phosphate residues by intestinal alkaline phosphatase could be a key point of that metabolism (Kibangou *et al*., 2005).

The soluble and colloidal phosphate fractions was significantly higher than that of the casein (Bosworth & Van Slyke, 1914) Casein contains about 0.71 per cent of phosphorus, the suggestion has been made that the lower figure is due to the splitting off of phosphorus from the casein molecule as the result of hydrolysis caused by prolonged contact with NH,OH (Van Slyke & Bosworth, 1915).

On the other hand, it was non-significant association between three groups genotype within prolactin gene polymorphism with serum phosphorus and serum calcium, this result was in agreement with (Williams *et al*., 1991) who found the dietary phosphorus level had no effect on phosphorus and calcium content of milk in the growing Angus

heifers, he also found saliva , blood, and rib bones of Phosphorus concentration reflected dietary Phosphorus additions.

Any significant differences were not noticed in the local cows in Karbala governorate on the level of calcium in their blood, the mean concentration Serum.

Generally, the mean concentration Serum of Calcium mg/dL in the blood serum of cross-breed cows was non-significant difference, it was found AA wild, AG heterozygote, GG polymorphism respectively, Table (4-4).

Generally, there is some study which indicated a direct relationship when calcium increases in the blood with a rise in the hormone prolactin (Ajibade *et al*., 2010), the prolactin modulates vitamin D-mediated calcium homeostasis, as well as prolactin has a direct effect on the 1α (OH)ase gene, which is mediated by STAT5 and JAK2. These mechanisms may have physiological importance during lactation (Ajibade *et al*., 2010).

The present study did not find an association ($p \geq 0.05$) between Kappa casein polymorphism with serum calcium in gross-breed cattle, Table (4-5).

The study refers to the explain the mineral content as a pronounced effect on the technological, properties of milk, as it affects its susceptibility, to renneting, fouling of heat exchangers, gelation and sedimentation. Of most interest are the free divalent cations, especially Ca2+, as they exist in the serum and can significantly influence the surrounding environment of the negatively charged casein micelles, and thus enhance or reduce the repelling forces between them (Tsioulpas *et al*., 2007).

Calcium ions are involved in the internal stability of casein micelles as they form linkages between the protein molecules either as CCP or directly bound to caseins (Bauland *et al*., 2020 and Lucey, 2016).

5.1.3. Hormonal analysis of cross-breed dairy cattle with prolactin and kappa casein genes polymorphism.

Otherwise, on the other hand, the mean concentration of Prolactin Hormone (PRL) mIU/L in the blood serum of cross-breed among Prolactin gene polymorphism has a significant ($P \leq 0.05$) difference. They found a decrease in GG polymorphism and in AG genotype compared with an increase in AA wild, respectively, as well as, the mean of serum concentration Prolactin Hormone (Prl) mIU/L in the blood serum of cross-breed was significant ($P \leq 0.05$) difference with kappa casein gene polymorphism it was increased in the AA wild compare with other two genotype AB heterozygote, BB mutation respectively, Table (4-6) and (4-7).

The mean serum concentration of Growth Hormone (GH) μIU/mL in the blood of cross-breed cows was non-significant differences in the Growth hormone μIU/mL with prolactin gene polymorphism. It found that AA wild, AG heterozygote, GG polymorphism, respectively, Otherwise, the Mean of serum concentration of Growth Hormone (GH) μIU/mL was non-significant differences in the growth hormone μIU/mL with kappa casein gene polymorphism. It found AA wild, AB heterozygote, BB polymorphism, respectively, Table (4-6) and (4-7).

The study revealed that during embryogenesis, the growth hormone-secreting cells (somatotrophs) and the prolactin-secreting cells (lactotrophs) develop from a common progenitor cell (somatomammotroph). Postnatally, in situations when one of these two

hormones is oversecreted, often the second hormone is, too (Laron, 2011). The polymorphisms of several genes including prolactin (PRL), growth hormone (GH) have been shown to affect milk yield and milk composition traits in dairy cattle (Ünal *et al*., 2015).

This result was not in agreement with (Zhou *et al*., 2008) who suggested that the fact of GH receptor mRNA and protein are expressed in the epithelial cells of the bovine mammary gland raise the possibility that GH might act directly on the mammary epithelial cells in cows to stimulate transcription of major milk protein genes, as part of the mechanism by which GH stimulates milk production. this difference is due to the sample size used in the study, as well as the health status of the animal, the isolation areas and the environmental condition

The mean serum concentration of Thyroid Stimulating Hormone (TSH) mIU/L in the blood serum of cross-breed cows was significant ($p \leq 0.05$) difference with prolactin gene polymorphism. It found increases in AA wild compared with AG heterozygote, and a decrease in the GG genotype, table (4-6). the hyperprolactinemia adversely affects the fertility potential by impairing pulsatile secretion of GnRH and hence interfering with ovulation (Goswami *et al*., 2009). This disorder was implicated in menstrual and ovulatory dysfunctions like amenorrhea, oligomenorrhea, anovulation, inadequate corpus luteal phase and galactorrhea (Sushilendu *et al*., 2020). However many infertile women present with normal menses despite a raised serum prolactin level. Pituitary hormones such as TSH, prolactin or growth hormone may act synergistically with FSH and LH to enhance the entry of non-growing follicles into the growth phase.

The role of thyroid hormones in milk is not yet understood; however, it is now possible to measure these hormones by extraction procedures followed by radioimmunoassay (RIA) (Akasha & Anderson, 1984).

At the same time, the mean serum concentration Thyroid Stimulating Hormone (TSH) mIU/L was significant ($P \leq 0.05$) difference with kappa casein gene polymorphism it was found increase in AA wild compare with decrease in the other two genotype AB heterozygote and BB polymorphism respectively table (4-7).

Thyroid hormones play a relatively important role in lactational processes (Graham, 1943a) observed that thyroidectomy of lactating cows decreased milk production markedly. The feeding of thyroactive compounds to dairy cows increased milk production (Reineke and Turner, 1942).

Study provided an-evidence for an involvement of S14 in mammary regulation of milk fat synthesis and a possible broader role for S14 in the reported antiobesity effects of CLA, SREBP1 and thyroid hormone responsive spot 14 (S14) are involved in the regulation of bovine mammary lipid synthesis during diet-induced milk fat depression and treatment with CLA (Harvatine & Bauman, 2006).

On the other hand , the mean concentration of Prolactin Hormone (PRL) mIU/L in the blood serum of cross-breed among Prolactin gene polymorphism was significant ($P \leq 0.05$) difference they were found decrease in GG polymorphism and in AG genotype compare with increase in AA wild, respectively, as well as, the mean of serum concentration Prolactin Hormone (Prl) mIU/L in the blood serum of cross-breed was significant ($P \leq 0.05$) difference with kappa casein gene polymorphism it was increased in the AA wild compare with other two

genotype AB heterozygote, BB mutation respectively table (4-6) and (4-7).

And our results were consistent with the results of the researcher (Patel & Chauhan, 2017) in terms of the effect of the PRL gene in milk yield (from the available pedigree data) showed the best mean value for the genotype AA , followed by AB and BB in Gir cattle breed . A similar result was also found in same study where Kankrej cattle with AA genotype cattle , followed by AB genotype cattle and BB genotype cattle produced milk, There was a significant difference between genotype AA and the other two (AB and BB genotype), thus determining the influence of the A allele in milk yield of both Gir and Kankrej cattle (B. indicus) ($P \leq 0.05$).

Prolactin is a protein hormone mainly, but not exclusively produced by lactotroph cells of the anterior pituitary. Its role in lactogenesis and galactopoiesis (maintenance of milk secretion) is well demonstrated (Bernichtein *et al*., 2010). Therefore, the gene encoding it (PRL) is considered to be one of the key links in the gene network constituting the hereditary component of milk productivity. Test systems for cattle breeding have been developed based on the associations of the PRL gene polymorphism with milk yield and quality (Lazebnaya *et al*., 2013).

Kobayashi *et al*., (2016) said that some of signal transduction protein like (Cldn3) in the region of TJs concurrent with less permeable TJ formation and high β-casein expression, and he was also found the inhibition of PRL secretion by bromocriptine in lactating mice induced the upregulation of Cldn3 the inhibition of Prolactin secretion by bromocriptine in lactating mice induced the upregulation of Cldn3 and Cldn4 concurrent with the downregulation of milk production.

Study has also found that there is a clear genetic heterogeneity between the prolactin gene and the casein gene in cow's milk, e.g. Akyüz & Ulas, (2014) who they studied the relationship between prolactine and kappa casein in four breed cattle in turkey, and others (Toparslan & Mercan, 2019) who was study the relationship between prolactin and kappa csein in water buffalo in Kızılırmak Delta. On the other hand, some study showed that there is a relationship between the prolactin gene and the casein gene in sheep milk in New York (Staiger *et al*., 2010).

The study measured Progesterone levels in serum of cattle to determine the association of progesterone concentration with Prolactin gene polymorphism, there were significant differences in the cattle have A allele in her blood ($P \leq 0,05$) table (4-6), it found (7.52) rather than other cattle groups, (7.93) and (4.51), respectively.

Estrogen and progesterone inhibit the stimulatory effects of prolactin on milk production, some of studies refers that progesterone lowered circulating prolactin levels significantly, these results indicate that a high level of progesterone in the luteal phase may partly block estrogen-induced prolactin release physiologically (Minakami *et al*., 1985).

Our result was found non-significant difference association between Prolactin gene polymorphism with Estrogen levels in cattle serum, it was found AA wild, AG heterozygote, GG polymorphism, respectively table (4-6).

A daily dose of 4 mg progesterone injected alone appeared to have no effect on serum and AP prolactin levels in the cross breed dairy cattle (Chen & Meites, 1970).

The study did not notice any significant differences between the kappa casein gene with the hormones progesterone and estrogen together. the

mean serum concentration of Progesterone Hormone (Pro) ng/mL in the blood serum of cross-breed cows was non-significant differences in the Progesterone ng/mL with kappa casein gene polymorphism, it was AA wild, AB heterozygote, BB polymorphism respectively, table (4-7), as well as, the mean serum concentration Estrogen (Estradiol) Hormone pg/mL in the blood serum of cross-breed cows was non-significant difference with kaapa casein gene polymorphism it was for AA wild, AB heterozygote, BB polymorphism respectively table (4-7), Close correlations between milk and plasma progesterone concentrations are found in most studies (Dobson and Fitzpatrick, 1976).

Our result found non-significant association between Prolactin gene polymorphism with Oxytocin levels in cattle serum, it was found increase in AA wild compare with AG heterozygote, GG mutation, respectively, table (4-6).

A great deal of literature has suggested that this prolactin-releasing factor may include oxytocin. Oxytocin receptors are present on lactotrophs. These oxytocin receptors respond to exogenous oxytocin and antagonism of endogenous oxytocin inhibits lactotroph activity (Kennett & McKee, 2012). In addition, the pattern of oxytocin neuronal activity and oxytocin release correlate with the release of prolactin. They are suggested that not only is oxytocin stimulating prolactin secretion, but we also hypothesize that prolactin secretion is controlled by a complex network of positive (oxytocin) and negative (dopamine) feedback loops (Egli *et al*., 2010) Oxytocin has several effects besides contracting the uterine wall during parturition and triggering the milk ejection reflex during lactation.

On the other hand, the mean serum concentration Oxytocin Hormone (OXY) mIU/L in the blood serum of cross-breed cows was significant ($P \leq 0.05$) difference with kappa casein gene polymorphism. It found an increase in AA wild compared with a decrease in two genotype AB heterozygote, BB polymorphism respectively table (4-7).

Oxytocin is a hormone released into the blood by neural stimulation that causes the contraction of myoepithelial cells, leading to the expulsion of milk from the alveoli. The mechanism of fast and complete milk removal from the udder is a complex one, normally not considered as one of the main factors of milk yield (Bruckmaier *et al*., 1994).

Sufficient literature is not available on the effect of oxytocin on protein milk production. Conclusively, Excessive use of oxytocin injections in cows for milking may disturb cell mechanisms for the synthesis of protein within mammary glands (Hameed *et al*., 2016).

5.1.4. Hematological analysis of cross-breed dairy cattle with prolactin and kappa casein genes polymorphism.

Our result found that mean level WBC x 10^9/L in the blood parameters of cross-breed cows was significant ($P \leq 0.05$) differences in the WBC with prolactin gene polymorphism, it was increase in AA wild compare with tow genotype AG heterozygote, GG polymorphism, respectively, table (4-8).

These results were in agreement with (Shukla *et al*., 2004) who conclude that complex partial or generalized seizures are associated with an increase in serum prolactin level. Peripheral WBC cell per 10^9 /L count increases significantly after a generalized seizure and is probably transient in nature.

The most important causes for the increase in WBCs in local gross-breed is social status management like ticks, fleas, lice or mites. There are some major problems and obstacles that accompany the animal during its social status, namely: lack of grazing land, lack of veterinary care and services, high prices and scarcity of feed and fodder (Mondal *et al*., 2010).

The study did not notice any significant differences .which are due to the relationship of the casein gene in the blood of cows and the blood parameters, except WBC. Mean level WBC x 10^9/L in the blood parameters of cross-breed cows was significant ($P \leq 0.05$) differences in the WBC with kappa casein gene polymorphism, it was found increase in AA wild compare with decrease in two genotype AB heterozygote, BB polymorphism for respectively, table (4-9).

Our result found non association significant with prolactin gene polymprphisim in the RBCs, Hb, MCV, MCH and MCHC among gross breed cattle in Karbala city it was demonstrated in the table (4-8).

Generally , the study found significant differences in the mean percentage HCT % in the blood parameters of cross-breed cows was significant ($P \leq 0.05$) differences in the (PCV%) with Prolactin gene polymorphism. there was an increase in AA wild compared with two genotype AG heterozygote, GG polymorphism respectively, table (4-8).

Part of study refers to hematocrit values that were observed to change during lactation and pregnancy, neither bodyweight nor milk production level were related to hematocrit readings. Seasonal changes were demonstrated (Fahrimal *et al*., 2020).

Otherwise, the mean level RBC x 10^{12}/L in the blood parameters of cross-breed cows was non-significant difference with kappa casein gene

polymorphism it was found 3.18, 3.02 and 3.14 for AA wild, AB heterozygote, BB polymorphism, respectively, table (4-9). on the other hand, , the mean Hb g/dL in the blood parameters of cross-breed cows was non-significant difference with kappa casein gene polymorphism it was AA wild, AB heterozygote, BB polymorphism, respectively table (4-9).

And the mean percentage (PCV %) in the blood parameters of cross-bred cows was non-significant differences in the HCT with kappa casein gene polymorphism, it was found AA wild, AB heterozygote, BB polymorphism, respectively, table (4-9), Otherwise, , the mean MCV fL in the blood parameters of cross-bred cows was non-significant difference with kappa casein gene polymorphism it was found AA wild, AB heterozygote, BB polymorphism, respectively table (4-9), on the other hand, the mean MCH pg in the blood parameters of cross-bred cows was non-significant difference with kappa casein gene polymorphism it was found AA wild, AB heterozygote, BB polymorphism, respectively table (4-9), Otherwise, the mean MCHC g/dL in the blood parameters of cross-bred cows was non-significant difference with kappa casein gene polymorphism it was found AA wild, AB heterozygote, BB polymorphism, respectively, table (4-9).

The cause of high white blood cells is not due to the casein gene, it may be due to insufficient control of the animal's nutrition or the difference in the animal's chain as well as the size of the female with the udder and other causes such as infectious agent (Detilleux *et al*.,1995).

5.2.1.Correlation between Prolactin gene polymorphism with milk production hormones concentration.

The result in the table (4.1) found a positive strong correlation (0.834) between thyroid stimulating hormone (TSH) with Progesterone hormones (PRO) among cattle have AA genotype, progesterone and thyroid hormones have a reciprocal relationship, some of study showed that progesterone can increase thyroid hormone levels in the blood, progesterone also decreases the amount of protein that carries thyroid in the blood so that more thyroid hormone can be free and get into the cells (Correa-Calderón *et al.,* 2021).on the other hand our results was noticed a positive mid-correlation (0.527) between Estrogen and growth hormone in the cattle have AA genotypes, There was a close interplay between estrogens and GH in the regulation of growth and development as exemplified in puberty. The increases in GH and estrogen trigger a growth spurt, which is accompanied by dramatic changes in physical development of cattle body (Devesa, & Caicedo, 2019).

In the table (4.11) the result not found any correlation between milk production hormones with Prolactin gene polymorphism for cattle have AG genotype in their blood.

The result found negative mid correlation (-573) between prolactin milk production hormones with progesterone in the cattle have prolactin gene polymorphism have GG genotype, progesterone action on prolactin levels in serum is observed with a normal brain-pituitary relationship and when high levels of estrogens are present, it is postulated that the negative feedback effect of progesterone on prolactin release by the pituitary is in part at the level of the brain (Bazer *et al*., 2009).

5.2.2.Analysis of milk production of cross-breed dairy cows with prolactin gene polymorphism.

The table (4.13) showed a significant difference ($p<0.05$) in milk quantity between three prolactin genotypes (AA, AG and GG) groups for three months . the genotype (GG) group result showed significant decrease it was different from other genotype ($p<0.05$) in milk quantity it was recorded as (19.94, 15.10 and 13.42) for three genotype in first month respectively and it was found increase in AA group compare with AG an GG

On other hand, our result revealed the genotype (AA) group increased significantly in milk production ($p<0.05$) in comparison with (AG) group and (GG) Group second month

as well as, it was notice found also there are increase significantly ($p<0.05$) in (20.11) for (AA) compare with AG and GG decreased in milk production in third month.

genes that are involved in mammary gland development, prolactin signalling and involution pathways are relevant candidates. Genes in the lactation pathway have been well-described but are largely inferred from mouse studies. Development of the mammary gland (or mammogenesis) involves the formation of the rudimentary mammary structure before puberty and is triggered by secreted signaling proteins and transcription factors that (Akers, 2006).

This result was agreement with (Collier *et al*., 1977) who was found an Effect of Reserpine on Milk Production and Serum Prolactin of cows Hormonally Induced into Lactation 1

Prolactin in serum increases dramatically a few days prepartum in pregnant cows (Convey, 1974), and suppression of prolactin by ergocryptine administration during this period partially inhibits lactation

(Dahl & Petitclerc, 2003). In vitro studies with explants of mammary tissue from pregnant cows have demonstrated that differentiation and the initiation of milk synthesis occurred when prolactin was added to explants cultured in a milieu containing insulin and a corticoid (Auchtung *et al*., 2005). Moreover, results of our investigations which characterized the changes in mammary tissue during the treatment period to induce lactation indicated tissue from the unsuccessful cow started to undergo differentiation but then appeared to regress.

5.3.1. Receiver operating characteristic for prediction of Prolactin in cattle.

Receiver operating characteristic (ROC) curve analysis was used to determine the best biochemical marker to predict prolactin concentration in serum cattle with area under curve (0.789) with 95% confidence interval 0.653 to 0.925. Animals with prolactin were considered affected if concentration less than 95 mIU/L , and those with an optimal amount of prolactin more than 100 mIU/L were considered as controls and not affected figure (4-1) (Nazifi *et al*., 2009).

The results from the ROC curve analyses used to examine the ability of biochemical parameters to detect Prolactin suggested that the best predictive markers for Prolactin deficiency were plasma PTH and serum Ca in the entire cohort and females (Velija-Asimi, 2014).

5.3.2.Receiver operating characteristic for prediction of kappa casein levels in cattle.

Cattle have A allele in the validation data set had an improved survival compared with cattles have B allele in the learning samples (figure 4-2). The median survival duration of Kappa casein is 2.546, 2.506 and 2.380, for AA wild, AB heterozygote, BB mutation, respectively figure (4-2).

The area under the ROC curve using the 120 cattle was .929 in the validation samples. Figure (4-2) presents how the predictions from the model at cattle with milk production have A allele compared with the actual survival probability for the cattle in our analysis(Yoshino *et al*., 2020).

5.3.3.Analysis of milk composition of cross-breed dairy cows with kappa casein gene polymorphism.

Our results were found insignificant for all milk component , except for protein and fat and freezing point, the percentage of protein was high in cows carrying the AA allele 3.72% , which is higher than the percentage of protein in cows carrying the allele AB and BB alleles, it was found as 3.132% and 3.183% respectively.

Kappa-casein is a protein yield and percentage gene that is significant in milk production. This test distinguishes between the two most frequent kappa-casein types, A and B, in the blood. Increased milk production is connected with both the A frequency and the AA genotype (Huppertz, 2013).

In addition, kappa-Casein is engaged in thiol-catalyzed disulfide exchange interactions with whey proteins during heat treatments, as well as in the facilitation of micelle coagulation after rennet cleavage and after

rennet cleavage. The three-dimensional structure of the protein on the micelle surface is responsible for these actions of kappa-CN (Li & Zhao, 2019). as shown in table (4.16).

We did not observe a significant increase in protein synthesis with kappa gene with local breed cows except for the gene that carries the A . allele

Hamza *et al*., (2010) The -casein gene consists of a 13 kb sequence that is separated into five exons. Alignment of point mutations in exon IV of the bovine kappa-casein (CSN3) gene results in the emergence of two allelic variants, designated A and B. The amino acids 136 and 148 are different between the A and B versions. For A and B, the amino acid threonine is substituted with the amino acid isoleucine at position 136, and the amino acid aspartic acid is substituted with the amino acid alanine at position 148. This variance, which is connected with processing characteristics such as cheese manufacturing technology, (Alipanah *et al* ., 2005).

This results can be investigated that a positive express of A allele on the milk and protein yield , otherwise it was less extended on fat yield. Fat percentage was lower because of the higher milk yield ,but nearly constant fat yield, associated with the A allele

Chapter Six

Conclusions & Recommendations

6.1.Conclusion

From the results obtained from our study, it could be concluded as follows :

1- Based on the statistical analysis, it is concluded that the most frequent genotype in Iraqi cattle population was AA (0.613) and GG genotype (0.46) for Kappa casein and Prolactin, respectively.

2- There was a significant decrease in serum PrL,TSH hormones in heterozygote alleles (AG, AB) and mutation (GG, BB) groups of prolactin and kappa casein gene polymorphism.

3- A significant decrease in milk quality in mutation genotype in two type of gene in our results prolactin gene and kappa casein gene polymorphism.

4- A significant decrease in serum iron in homozygous mutation, heterozygote groups of prolactin and kappa casein gene polymorphism.

5- A significant decrease in protein, and fat percentage in prolactin and kappa casein gene polymorphism in AG, AB, GG and BB groups.

6.2.Recommendation

1- Future study about the effect of Prolactin and Kappa casein gene polymorphism on some biochemical parameters in buffalo .

2- Future study about the effect of another genes on milk production as proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) and cytochrome P450 family 11 subfamily B hydroxylase (CYP11B1).

3- Real-Time PCR technique should be used to identify the gene expression of prolactin and Kappa casein protein levels.

4- studying the feeding of low-production animals in order to improve the high production quantity.

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Appendix

Appendix 1

Animal choosing

One hundred twenty apparently healthy dairy crossbred cows were used in this study , their aged ranged from (4-6) years ,and weight ranged from (278 – 305 KG) in mid stage (40-120) days of lactation, according heart girth equation (Wangchuk et al., 2018) to the obtained from unorganized fields of rural area in different region of Karbala city during the period from (December 2020 - May 2021)

Appendix 2

Collection of Milk Samples

30ml of milk have been taken from lactation cattle and collected in sterile disposable cup and stored in ice box at (4C) until the milk was analyzed by (EKOMILK Ultrasonic milk analyzers) in public health laboratory in veterinary medicine college of AL-Kuffa university.

Appendix 3

DNA Extraction

3 ml of blood in EDTA tubes was used to extract DNA by using Mini DNA extraction Kit with whole Blood Protocol (Appendix 3) according to the manufacture company.

PROTOCOL A (for Blood, body fluids)

1.Pipet 200 µl of whole blood or body fluids into a 1.5 ml microcentrifuge tube (not provided). Note : If the volume of sample is less than 200 µl, use Buffer CL or PBS Buffer

2.Add 20 µl of Proteinase K and 5 µl of RNase A Solution into sample tube and gently mix. Note : It is possible to add Proteinase K to blood sample that have already been measured into 1.5 ml tube. It is important to ensure proper mixing after adding the Proteinase K and RNase A solution.

3. Add 200 µl of Buffer BL into upper sample tube and mix thoroughly. Note : In order to ensure efficient lysis, it is important that the blood sample and Buffer BL are mixed thoroughly to yield a lysis solution.

4.Place the mixture at Room Temperature for 2minutes.

5.Incubate the lysate at 56°C for 10 min. Note : For complete lysis, mix 3 or 4 times during incubation by inverting tube. If it lysis perfectly, the red color of lysate becomes the dark green.

6.Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.

7.Add 200 µl of absolute ethanol into the lysate, and mix well by pulse vortex. After mixing, briefly centrifuge the 1.5 ml tube to remove drops from inside of the lid. Note : This step is an equilibration step for binding genomic DNA to column membrane. It is important to assure proper mixing after adding the ethanol, until not showing 2-phase which is not mixed. Also, this step conduces to pass efficiently cell lysate through a column.

8.Carefully apply the mixture from step 7 to the Spin Column (in a 2 ml Collection Tube) without wetting the rim, close the cap, and centrifuge at 13,000 rpm for 1 min. Discard the filtrate and place the Spin Column in a new 2 ml Collection Tube (additionally supplied). Note : Close each Spin Column in order to avoid aerosol formation during centrifugation. Do not transfer any solid materials.

9.Add 700 µl of Buffer WA (Buffer WB) to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and reuse the Collection Tube. Note : Use Buffer WB for cell sample.

10.Add 700 µl of Buffer WB to the Spin Column without wetting the rim, and centrifuge for 1 min at 13,000 rpm. Discard the flow-through and place the Column into a new 2.0 ml Collection Tube (additionally supplied), Then again centrifuge for additional 1 min to dry the membrane. Discard the flow-through and Collection Tube altogether. Note : It is very important to dry the membrane of the Spin Column since residual ethanol may inhibit subsequent reactions. Following the centrifugation, remove carefully the Spin Column from the Collection Tube without contacting with the flow-through, since this will result in carryover of ethanol. .

11. Place the Spin Column into a new 1.5 ml tube (not supplied), and add 30 - 100 µl of Buffer CE directly onto the membrane. Incubate for 1 min at room temperature and then centrifuge for 1 min at 13,000 rpm to elute. Note : In general, Elution with 30 µl (instead of 50 µl) increases the final DNA concentration, but reduces overall DNA yield. **Note** : A new 1.5 ml tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, the tube can be reused for the second elution step to combine the eluates.

Appendix 4

Hormonal analysis:

The serum gel tubes were centrifuged at 2500 xg for 10 minutes, separated and divided into two aliquots and stored at (–20 C) until time of use for hormones estimation (PRL, OXY, GH, TSH, Es and PRO hormones) by using Enzyme linked ImmunoSorbent Assay **(ElISA),** According formation of kits appendix 4 (1,2,3,4,5,6).

Procedure

1. Dilution of Standards

Ten wells are set for standards in a Microelisa stripplate. In Well 1 and Well 2, 100µl Standard solution and 50µl Standard Dilution buffer are added and mixed well. In Well 3 and Well 4, 100µl solution from Well 1 and Well 2 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is discarded from Well 3 and Well 4. In Well 5 and Well 6, 50µl solution from Well 3 and Well 4 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 7 and Well 8, 50µl solution from Well 5 and Well 6 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 9 and Well 10, 50µl solution from Well 7 and Well 8 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is discarded from Well 9 and Well 10. After dilution, the total volume in all the wells are 50µl and the concentrations are 18 pg/ml，12 pg/ml ，6pg/ml，3pg/ml and 1.5pg/ml, respectively.

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added

(dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubation: incubate 30 min at 37℃ after sealed with Closure plate membrane.

4. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).

5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Add 50 μl HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washing as described in Step 5.

9. Coloring: Add 50 μl Chromogen Solution A and 50 μl Chromogen Solution B to each well, mix with gently shaking and incubate at 37 ℃ for 15 minutes. Please avoid light during coloring.

10. Termination: add 50 μl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11.Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Appendix **5**

Milk analysis:

Information on milk yield were gained from unorganized farms for each cow (Appendeix 5) with exclusion of cows which have some criteria mentioned later. whole morning and evening milk were collected during milking into a sterile bucket and the milk weight was determined with a spring scale.10ml of milk samples were measured by EKOMILK Ultrasonic milk analyzers, Ultrasonic milk analyzer system was determine the milk components percentage were run to obtained an average of parameter by infrared spectrum for milk samples according to the value of absorbance a each wavelength the concentrations of fat, protein, lactose and SNF.

Appendix 6

Body weight measurement :

A prediction equation for cattle body weight was estimated and calculated

according to the following formula :

W=(L.G2)/300

W: is body weight of animal in pounds

L: is the length from point of shoulder to pin bone measured in inches

G: is the chest girth of the animal in inches figure (3.3) .

The final weight was converted from pound to kg by multiplication in 0.4536) (Wangchuk *et al*., 2018)

Appendix 7

Statistical analysis

All the data were written in Excel sheet , the experience was design according to the Complete Randomly Design(CRD) ,the results were analyzed using Statistical Software (SAS Institute 2002 Ver. 9) and arithmetic averages were measured on Duncan Multidimensional scale (Duncun,1955).

The Frequencies of the Prolactin gene polymorphisms AA, AG and GG and Kappa casein gene Polymorphisms AA, AB. BB were expressed in numbers and percentages for wild-type, recessive and heterozygosity genotypes. $\chi 2$ test was used to evaluate consistency of genotype distributions with Hardy-Weinberg equilibrium.

Appendix 8

Animal information

Cow No. :

Time:

Date :

Owner, s name & address:

Telephone :

Location :

Age :

Weight :(L)= inch. (G)= inch

Amount of milk /kg :

Morning kg/day:

Afternoon kg/day:

No. of calving :

Sex of calve :

Age of calve :

No of lactation :

Nutrition :

Previous treatment :

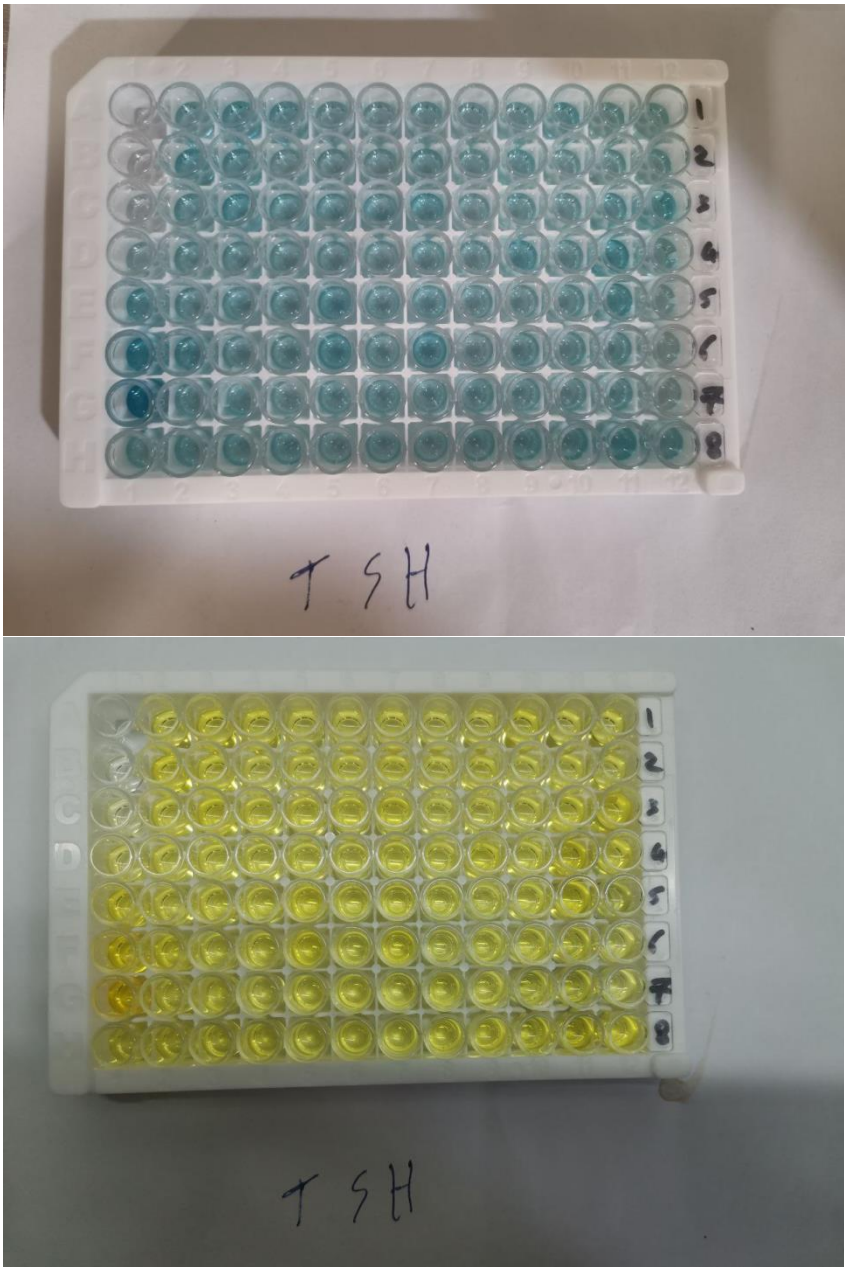
Sample → Blood

↘ Milk

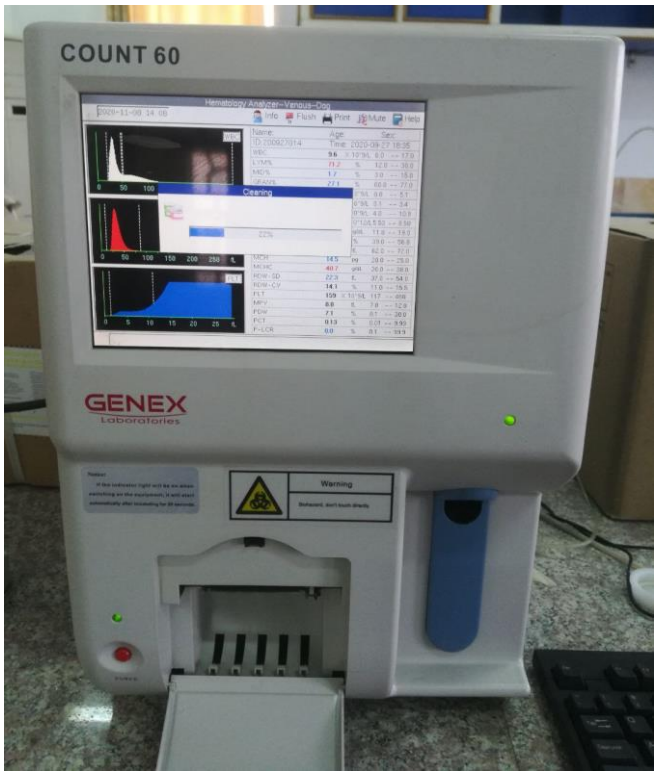
Percentage of milk coagulant :

Appendix 9

A sample test of Bovine growth hormone ELISA Kit

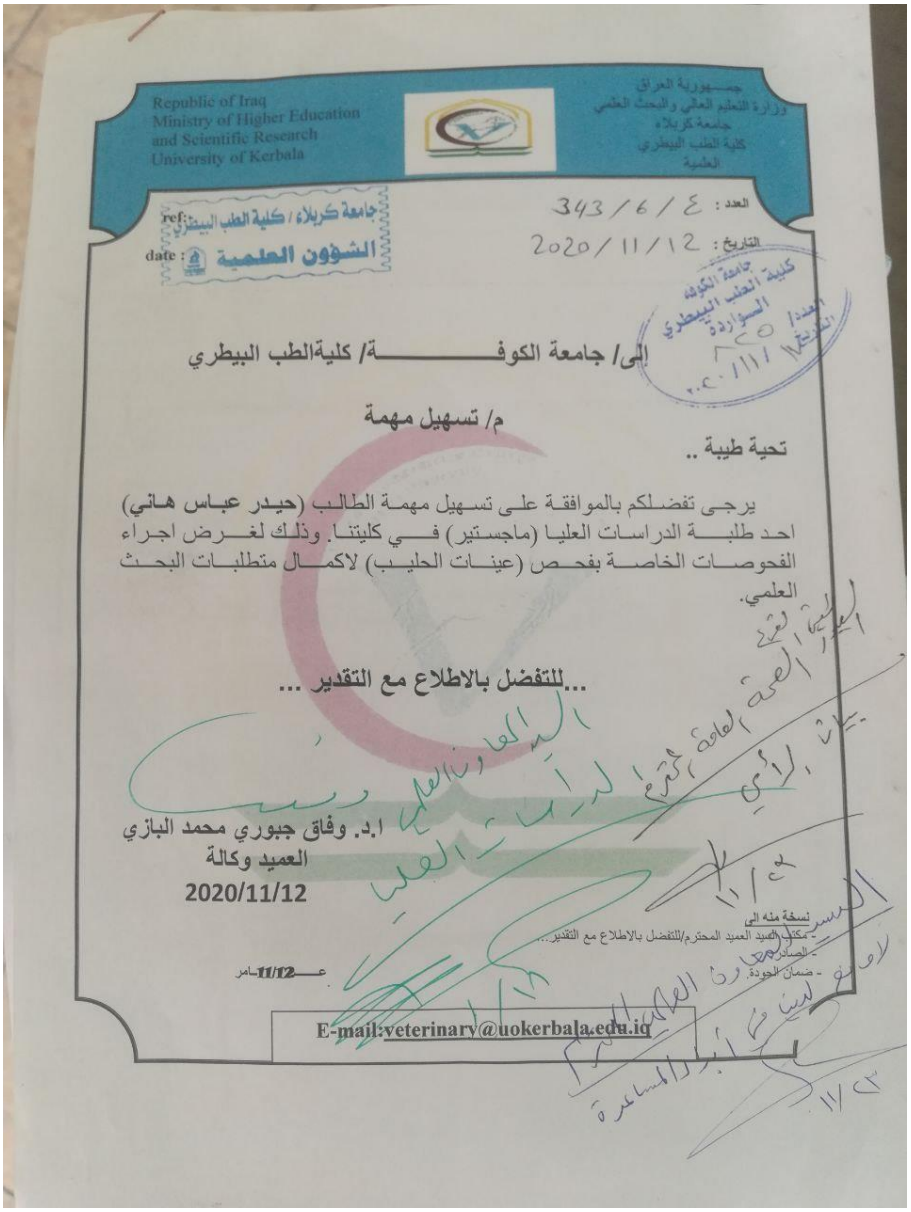


Appendix 10



Eko milk scan

Appendix 11



جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الطب البيطري
العلمية

Republic of Iraq
Ministry of Higher Education
and Scientific Research
University of Kerbala

العدد : ٤/٦/٣٤٣
التاريخ : 2020/11/12

ref: جامعة كربلاء / كلية الطب البيطري
date : الشؤون العلمية

جامعة الكوفة
كلية الطب البيطري
الموارده
العدد/ ٢٥٠
التاريخ/ ١١/ ٢٠٢٠

إلى/ جامعة الكوفـــــــــــة/ كليةالطب البيطري

م/ تسهيل مهمة

تحية طيبة ..

يرجى تفضلكم بالموافقة على تسهيل مهمة الطالب (حيدر عباس هاني) احد طلبة الدراسات العليا (ماجستير) في كليتنا. وذلك لغرض اجراء الفحوصات الخاصة بفحص (عينات الحليب) لاكمال متطلبات البحث العلمي.

...للتفضل بالاطلاع مع التقدير ...

ا.د. وفاق جبوري محمد البازي
العميد وكالة
2020/11/12

نسخة منه الى
- مكتب السيد العميد المحترم/للتفضل بالاطلاع مع التقدير...
- الصادر
- ضمان الجودة

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الخلاصة :

أجريت هذه الدراسة لمعرفة دور تعدد أشكال جينات البرولاكتين وكابا الكازين المرتبطة بصفات الحليب وبعض تركيز الهرمونات ، وكذلك التحقق من تأثير تعدد أشكال جينات البرولاكتين والكابا على جودة الحليب وكمية تركيز المعادن مثل البروتين واللاكتوز. ونقطة التجمد والكثافة والدهون والصلب غير الدهني وكزين ، ومع وبعض العناصر النزرة مثل مصل الحديد (Fe) ، مصل الفوسفور (P) ومصل الكالسيوم (Ca) ، ومع تركيز بعض الهرمونات مثل البرولاكتين (PRL) ، وهرمون النمو (GH) ، وهرمون تحفيز الغدة الدرقية (TSH) ، وهرمون البروجسترون (PRO) ، وهرمون الاستروجين (Es) ، وهرمون الأوكسيتوسين (OXY) ، وكذلك التحقق من تأثير تعدد الأشكال في جينات البرولاكتين والكابا الكازين على بعض مقاييس الدم مثل: عدد خلايا الدم الحمراء (RBC) ، حجم الخلايا المعبأة (PCV) ، الهيموجلوبين الملوث (Hb) ، متوسط حجم الكريات الحمر (MCV) ، الهيموجلوبين العضلي المسبب للسرطان (MCHC) ، أبقار الألبان لخلايا الدم البيضاء (WBC) في محافظة كربلاء.

هذه الدراسة هي الأولى من نوعها في العراق. تم استخدام مائة وعشرون بقرة حلوب سليمة لجمع عينات الدم والمصل في فترة الرضاعة في المرحلة (40-120) يوما خلال شهر كانون الأول 2020 حتى أيار 2021 في المناطق الريفية الشمالية في محافظة كربلاء في العراق.

تم تصنيف عينة الدم بعد اكتشاف تعدد الأشكال لجين البرولاكتين وكابا الكازين باستخدام تقنيات نظام الطفرة الحرارية التضخمية (ARMS- PCR) وتقنيات RFLP-PCR لثلاث مجموعات من الأنماط الجينية لتعدد الأشكال الجيني البرولاكتين كمجموعة برية (AA) ، مجموعة متنحية (AG) ومجموعة تغاير الزيجوت (GG) ، وكذلك تعدد الأشكال لجين كابا كازين لثلاثة طرز وراثية إلى AA البرية ومجموعة AB المتنحية ومجموعة BB غير المتجانسة.

كانت نتيجتنا هناك فروقات معنوية في نسبة البروتين واللاكتوز والدهون والصلبة غير الدهنية وكثافتها في AG و GG و AB و BB المقارن مع مجموعة النمط الجيني Wild.

من ناحية أخرى أظهرت النتائج الحالية أيضًا زيادة معنوية ($p\leq 0.05$) في تركيز المصل في الحديد مع مجموعة AA مقارنة مع AG، GG، AB، BB بينما في مصل الفسفور زيادة في مجموعات AA من تعدد الأشكال الجيني كابا كازين مقارنة مع لم تكن مجموعات AB و BB وفي تركيز الكالسيوم في الدم معنوياً في تعدد الأشكال الجيني البرولاكتين والكازين.

أظهرت نتائجنا انخفاضًا معنويًا ($p\leq 0.05$) في تركيز المصل في هرمونات PRL و TSH و Pro في مجموعات AG و GG مقارنة بالمجموعات البرية من تعدد أشكال جينات البرولاكتين بينما لا يوجد فرق معنوي في GH و Esr و OXY في AG و GG مقارنة مع مجموعة AA بينما كانت هناك مراسيم معنوية في TSH في AB و BB مقارنة بمجموعة AA من تعدد الأشكال لجين الكابا كازين ، كما لا توجد معنوية في GH و Pro و Esr و OXY لتركيز مجموعة AB و BB مقارنة بمجموعات AA.

أظهرت النتيجة الحالية أيضًا عدم وجود فرق معنوي في Hb و PCV و RBC و MCV و MCHC في جميع مجموعات تعدد الأشكال الوراثي - كما أظهرت الدراسة ارتباطًا إيجابيًا بين تركيز TSH مع تعدد الأشكال الجيني البرولاكتين في مجموعة النمط الجيني AA و GH مع هرمون الاستروجين في مجموعة AA.

أستنتجت الدراسة الحالية النتائج بأن تعدد الأشكال الجيني البرولاكتين كان له تأثير على تركيز هرمون البرولاكتين ، TSH وهرمونات البروجسترون وكمية ونوعية إنتاج الحليب ، بينما لم يؤثر تعدد الأشكال الجيني كابا كازين على نسبة بروتين الكازين في أبقار الألبان الهجين في محافظة كربلاء.

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دراسة علاقة تعدد أشكال جيني البرولاكتين والكابا كازيين كلآ على حدة في بعض الصفات الفسيولوجية وأنتاج الحليب في الابقار المحلية في محافظة كربلاء

رسالة مقدمة الى

مجلس كلية الطب البيطري - جامعة كربلاء وهي جزء من متطلبات نيل

درجة الماجستير في علوم الطب البيطري / الفسلجة

من قبل

حيدر عباس هاني الحسناوي

بكالوريوس طب وجراحة بيطرية

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