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DETERMINATION OF AFLATOXIN IN RAW AND PASTEURIZED MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN CENTRAL ETHIOPIA

MVSC THESIS

BY:

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DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

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DETERMINATION OF AFLATOXIN IN RAW AND PASTEURIZED MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) IN CENTRAL ETHIOPIA

BY:

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

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Bishoftu, Ethiopia.

Addis Ababa University

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As MVSc research advisors, I here certify that I have read and evaluated the Thesis prepared by **Sitena Kebede under my guidance entitled as: "Determination of Aflatoxin in Raw and Pasteurized Milk by High Performance Liquid Chromatography (HPLC) in Central Ethiopia";** I recommend that it can be submitted as fulfilling the MVSc Thesis requirement.

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STATEMENT OF AUTHOR

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

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LIST OF ABBREVIATIONS

AFT	Aflatoxin
AFM1	Aflatoxin in milk
ELISA	Enzyme-Linked Immunosorbent Assay
LC	Liquid Chromatography
TLC	Thin-Layer Chromatography
HPLC	High Performance Liquid Chromatography
UV	Ultra- Violet
IARC	International Agency for Research on Cancer
WBC	White Blood Cell
RBC	Red Blood Cell
APVD-FQAC	Animal Product, Veterinary Drug and Feed Quality Assessment Center
FLD	Fluorescence Detect

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ABSTRACT

A cross-sectional study was conducted from October 2020 to May 2021 with the aim of detecting and quantifying the amount of aflatoxin M1(AFM1) in raw and pasteurized milk in central Ethiopia by high performance liquid chromatography (HPLC) using C18 column with fluorescence detector. The mobile phase was water-acetonitrile-methanol (60:25:15V/V/V) at flow rate of 1ml/minute. The HPLC instrument was conditioned with working standard solution of different concentration (0.05 to 4µg/l) to get the calibration curve. The obtained linearity(r) of concentration with their peak area was 0.99937 and the coefficient of determination (r^2) was 0.99875. The study was conducted on total of 114 cow milk samples consisting of 60 raw milk and 54 pasteurized milks. From the total 114 tested milk sample AFM1 was detected on 79(69.3%) of them. From those positive samples 25.4% of them contain AFM1 above the maximum limit of EU (0.05 µg/l) and 1.8% of them contain above the maximum limit of FDA (0.5 µg/l). The maximum and mean concentration were 0.893 and 0.0465 respectively. The study result shows significant difference between contamination level of AFM1 in raw milk with considered risk factors (storage time, presence of noug in feed, using grazing or not). Higher contamination of AFM1 was detected in pasteurized milk (96.3%) than raw milk (16.7%). 35.2% of pasteurized milk and 16% of raw milk was contain AFM1 above the maximum limit of 0.05ug/l. There was no significance difference between brands of pasteurized milk which was considered in this study. Which means, pasteurization didn't remove aflatoxin from milk. Site of sample collection have significant difference. 26.7% of sample from Sebata and 6.7% from sululta contain above the maximum limit (0.05 µg/L). In conclusion, AFM1 concentration level both in raw and pasteurized milk was not safe for human consumption. Due to its heat resistant properties AFM1 found in pasteurized milk which pose great public health risk both for children and adults. Thus, awareness creation on feed management practice of farmers, because animal feed is the main source of aflatoxin and risk mitigation method is very crucial to reduce its public health threat.

Key words: ***AFM1, Dairy Farm, HPLC, Raw milk, Pasteurized milk, Sebata, Sululta***

1. INTRODUCTION

Aflatoxins are a group of structurally related mycotoxins produced by certain species of fungus in the genus *Aspergillus*, particularly *Aspergillus flavus (A. flavus), Aspergillus parasiticus (A. parasiticus) and Aspergillus nomius (A. nomius)*. It was first discovered in 1960 when about 100,000 turkey were died in UK and the cause was identified as *Aspergillus flavus*, the name is also given from its cause(Bennett *et al*., 2007). Aflatoxins are produced as a secondary metabolite and the most commonly known are: B1, B2, G1, G2, M1 and M2. The letter B and M stands for their colour under florescence detection where, B is for blue colour and G is for green colour. Those M1 and M2 are metabolite of B1 and B2, respectively. M1 and M2 are found in food of animal origin like milk and milk products, meat and eggs(Jaimez *et al*., 2000). AFB1 is well known and most prevalent toxin, its targeting organ is liver and has teratogenic, mutagenic and carcinogenic effects in both animals and humans. The level of toxicity is AFTs-B1 > AFTs-G1 > AFTs-B2 > AFTs- G2(Ismail *et al*., 2015). Aflatoxin M1 (AFM1) is four times hydroxylated and produced by hepatic biotransformation of AFB1 in liver of animals consuming feed contaminated with AFB1 and secreted in milk. The extent of contamination depends on the season, (winter being more than summer), environmental and genetic condition of the animal(Fallah *et al*., 2011;Abyaneh *et al*., 2019).

About 0.3% to 6.2% of AFB1 is converted to AFM1 and classified as cause of human cancer especially liver cancer. Excretion of AFM1 in milk can take time from 12 - 24 hours after the ingestion of AFB1, however, there is a decrease in concentration after 72 hours (Fallah *et al*., 2011; Alahlah *et al*., 2020;Bukari *et al*., 2020).

Milk and milk product are one of the primary important diet of human around the world and presence of AFM1 in milk can cause serious health problem to human. AFM1 is classified as group one causative agent of human cancer by International Agency for Research on Cancer(IARC) (Sharma *et al*., 2019). The occurrence of aflatoxin in human, animal and milk products is one of the most serious health problem of food safety since milk is important food for adults, and the unique nutrient for infant (Polak-Śliwińska, 2020).

Aflatoxin M1 (AFM1) has a potency which is close to that of the potency of aflatoxin B1 (AFB1). Therefore, it is important to determine Aflatoxin M1 levels in milk to protect children and adult from its potential health hazards(Dehcheshmeh *et al*., 2020). AFM1 is resistant to autoclaving, pasteurization and thermal inactivation. AFM1 has both acute and chronic effect(Sani and Nikpooyan, 2013).

Concerning its serious health effect, many countries set limit for its presence in feed and foods. commission regulation of European Union (EU) set that the maximum level of AFM1 in liquid milk should not exceed 0.05ug/l(ppb). and the US standard which set by Food and Drug Administration (USFDA) indicate that it should not to be higher than 0.5ug/l in liquid milk(Markaki and Melissari, 1997; Maqbool *et al*., 2009). Aflatoxin is a major food safety problem in tropical and subtropical area due to environmental condition but it is not limited to those areas, there is also a report from temperate environment and worldwide.

Ethiopia produces approximately 3.2 billion liters of milk per year(Desalegn, 2018). But there are different factors which affect its safety and have health risk to the consumer. The presence of aflatoxin in milk is one of critical issue which need due attention to safeguard the public. The previous study by Gizachew and his colleagues in Addis Ababa milk shade area was report high contamination level of AFM1 in milk and at the time it was great national issue and increase awareness of people on aflatoxin(Gizachew *et al*., 2016).

Then after there was no report about the status or contamination level of AFM1 in central Ethiopia including the current study areas, (Sebata and Sululta in North Showa zone of central Ethiopia). So, there is a need to do further study on the current status of AFM1 in central Ethiopia designated as major Addis Ababa milkshed areas. It is known that Aflatoxin is not completely removed by pasteurization and other thermal treatment, but so far there is no report on contamination level of AFM1 in pasteurized milk in Ethiopia which initiate this study and will be addressed for the first time. Moreover, the current study used HPLC laboratory analysis method for detection and quantification of the contamination level of AFM1 in milk which is more accurate and precise.

Therefore, the objectives of this study were: -

- To detect and quantify contamination level of AFM1 in raw cow's milk in selected Addis Ababa milkshed areas of central Ethiopia specifically in Sebata and Sululta area.
- To detect and quantify contamination level of AFM1 in pasteurized cow's milk from three different brands produced by milk processing plants located in central Ethiopia.
- To investigate the association between potential risk factors and AFM1 contamination level in milk and to asses knowledge and attitude of farmers on aflatoxin

2. LITERATURE REVIEW ON AFLATOXIN

2.1. Aflatoxins

Aflatoxins (AFs) are one of the first recognized and vastly researched mycotoxins in the world. They are one of the most potent toxic substances produced by some of fungi species of genus *Aspergillus*, such as *Aspergillus flavus, A. parasiticus and rarely by A. nomius*. They are ubiquitous fungus and they produce Aflatoxin as a secondary metabolite (intermediates and products of metabolism) under favorable condition for toxin formation (Ghiasian and Maghsood, 2012). Aflatoxin is well known and most common natural mycotoxin contaminants. The name (Aflatoxin) is composite word of A, to indicate genus aspergillus, fla, to indicate flavus and toxin as it is toxin product. A. flavus produces only B aflatoxins, while the other two species produce both B and G aflatoxins. Aflatoxins M1 and M2 are the hydroxylated metabolites of aflatoxins B1 and B2 and can be found in milk or milk products obtained from dairy cows that have ingested contaminated feed. Known sources of aflatoxins in feeds are peanut meal, maize, noug seed cake and cottonseed meal (Dhanasekaran *et al*., 2011). Aflatoxicosis is the disease/poisoning that results from ingesting aflatoxins either from food or from milk and milk product. It can be acute severe intoxication, which results in liver damage, subsequent illness and death, and the other one is chronic sub symptomatic exposure which result in liver cancer(Fedlu *et al*., 2019).

Chronic aflatoxicosis is result from long-term exposure to low levels of aflatoxins from contaminated food and milk. Exposure to moderate levels of aflatoxin has been linked to development of liver cancer, impaired growth, immunosuppression and other secondary complication(Atherstone *et al.*, 2014).

2.2. Taxonomy

Aflatoxin causing agent are belong to kingdom *Fungi*, phylum *Ascomycota*, order *Eurotiales*, class *Eurotiomycetes*, family *Trichocomaceae*, genus *Aspergillus*, species *flavus, parasiticus, nomius* and others. Aspergillus is an anamorphic genus consisting of about 250

recognized species. It is characterized by a distinctive spore-bearing structure, the aspergillum(Klich, 2007).

Genus Aspergillus *contain* the major economically important aflatoxin-producing fungi. Those are: A. *flavus and A. parasiticus. Aspergillus flavus* is fast-growing yellow–green colonies and 65–70 mm in diameter after 7 days growth in the dark at 25 °C on Czapek yeast extract (CYA) media. It grows well at temperature of 37 °C. *Aspergillus parasiticus* colonies are generally darker green, and the conidial walls are very(Klich, 2007).

2.3 Epidemiology

Aspergillus flavus is a weak pathogen that infects plants, animals and humans. When it infects agricultural crops, however, it produces one of the most potent carcinogens toxin known as aflatoxins which is becoming major food borne mycotoxins(Yu *et al*., 2008). It can affect as wide range of food commodities and occur widely, in temperate, sub-tropical and tropical climates, throughout the world and aflatoxins can be produced, both before and after harvest and also during storage, on many foods and feeds especially oilseeds, edible nuts and cereals. Although aflatoxins are predominantly associated with commodities of sub-tropical and tropical origin, their occurrence has also been reported in temperate climates in acid-treated grains and (Ketney *et al*., 2017).

2.4. Risk factors

Physical factors for aflatoxin production include favorable temperature, pH, light, moisture, relative humidity, water and level of atmospheric gases. Aflatoxins are produced between temperatures of 12-42°C and the optimal temperature is 25-35°C and the moisture content is above 7% (10% with ventilation). The range of pH is (1.7-9.3) with an optimum of around 3-7 PH. Generally, at lower temperatures yield of AFB and AFG are in equal amounts but at higher temperatures AFB production becomes dominant. High temperatures and drought stress affect the physiology of plants, and therefore stressed plants may be more susceptible to infection or aflatoxin production (Klich, 2007; Bellio *et al*., 2016).

Substrate and nutrients of the food play an important role in aflatoxin production. The amount of aflatoxin formed depends on the type of substrate. Aflatoxin production is greater in carbohydrate food than in oil seeds. It is affected by the identity and concentration of available carbon source. Glucose, ribose, sucrose, xylose and glycerol were considered as excellent substrates for aflatoxin production(Abrar *et al*., 2013).

Figure 1. Cause and consequence of aflatoxin. Source (Cambaza et al., 2018).

2.5 History of aflatoxin

Acute aflatoxicosis in animals was first documented in 1960 after more than 100,000 turkeys died due to outbreak in the United Kingdom. During the investigations moldy feed toxicosis which was called Turkey "x" disease, Aspergillus flavus and A. parasiticus were identified as the organisms responsible for the elaboration of the toxin in the feed. It was associated with the Brazilian groundnut feed (Farombi *et al*., 2009). An intensive study of that groundnut meal revealed its toxic nature as it produces similar symptoms of Turkey-X disease when consumed by poultry and ducks. A study on the nature of the toxin suggested its origin from the fungus Aspergillus flavus. Thus, the toxin was named "aflatoxin" by virtue of its origin from Aspergillus Flavus. Research on aflatoxins led to a "golden age" of mycotoxins research and result in several discoveries of new mycotoxins. Other important mycotoxins produced by

Aspergillus are, Fusarium and Penicillium include ochratoxin, patulin and fumonisins(Bennett *et al*., 2007). From those mycotoxins and polyketide compounds synthesized by fungal species, aflatoxins (the most potent hepatotoxic and carcinogenic metabolites) continue to receive major attention and it is intensely studied(Dhanasekaran *et al*., 2011).

The outbreak of acute hepatotoxicity was identified in Kenya's eastern and central provinces. Epidemiologic investigations determined that the out- break was due to aflatoxin poisoning from ingestion of contaminated maize. As of July 2004, 317 cases and 125 deaths had occurred and it was the largest and severe outbreaks of acute aflatoxicosis documented in the world(Farombi *et al*., 2009).

2.6. Physical and Chemical properties of aflatoxin

Aflatoxins are crystalline odorless and solids when isolated and their color range from pale white to yellow. They have closely related structures and form unique groups which are highly oxygenated and heterocyclic compounds. The two major types of aflatoxin were named as aflatoxins B and G (blue and green) after the color of their fluorescence under longwave ultraviolet light. This intense fluorescence forms the basis of most assay techniques for aflatoxins (Vijaya Kumar, 2018). They are unstable under UV light in presence of oxygen. These B1, B2, M1, M2 compounds were isolated by groups of investigators in England. They can Intensely fluorescent under ultraviolet light and emit blue (aflatoxins B1 and B2) or green (aflatoxin G1), blue–violet fluorescence (aflatoxin M1) and green–blue (aflatoxin G2) (Bennett *et al*., 2007;Dhanasekaran *et al*., 2011).

Chemically, aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway(Klich, 2007). They are slightly soluble in water and freely soluble in moderately polar solvents such as methanol, chloroform, dimethyl sulfoxide, acetone and acetonitrile. Aflatoxins react with alkaline solutions causing the hydrolysis of the lactone's moiety. The lactone ring opens under alkaline conditions and Aflatoxin is destroyed, but this reaction is reversible on acidification. Ammoniation cause opening of lactone ring at high

temperature and result in decarboxylation of aflatoxins and this reaction is irreversible (Vijaya Kumar, 2018; Balina *et al*., 2018).

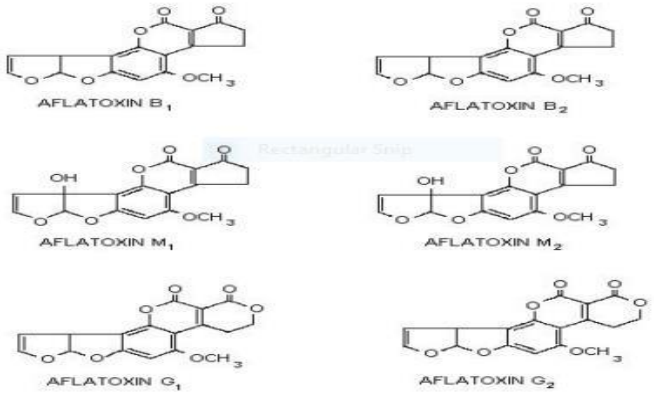

Figure 2. Structure of major aflatoxins adopted from (Dhanasekaran et al., 2011).

About 0.3% to 6.2% of the ingested AFB1 is converted to the monohydroxy derivative aflatoxin M1 in the liver of lactating animals, by the action of cytochrome P 450, and is secreted in milk. When aflatoxinB1and B2 is hydrated they can be converted into aflatoxin M1 and M2 respectively(Min *et al*., 2021). Due to the binding of AFM1 to the milk proteins, particularly with casein, it can be present in dairy products, such as cheese and yoghurt (Balina *et al*., 2018; Vaz *et al*., 2020).

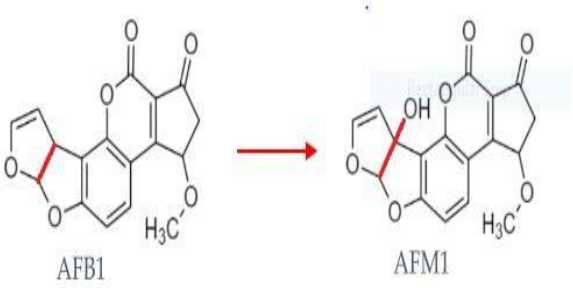

Figure 3. Conversion of aflatoxin AFB1 to AFM1 source (Vaz et al., 2020).

2.7. Mechanism of Action of Aflatoxins

The absorption from the gastrointestinal tract should be complete since very small doses, even in the presence of food, can cause toxicity. After the absorption, highest concentration of the toxin is found in the liver. Once in liver, aflatoxin B1 is metabolized by microsomal enzymes to different metabolites through hydroxylation, hydration, demethylation and epoxidation. Hydroxylation's of AFB1 at C4 produce AFM1. Liver is the target organ for toxic effects of aflatoxin B1. As a result, metabolism of proteins, carbohydrates and lipids in liver is seriously impaired by AFB1. The toxin inhibits RNA polymerase and subsequent protein synthesis(Dhanasekaran *et al.,* 2011).

Aflatoxins are easily absorbed across cell membranes from the site of exposure such as gastrointestinal, respiratory tracts and then enter into the blood stream, then spread to various tissues and to the liver. They are metabolized in liver to reactive epoxide intermediate or hydroxylated to less toxic aflatoxin M1(Shan, 2020). In humans and susceptible animals, cytochrome P450 microsomal enzyme converts AFB1 to an epoxide which binds to DNA and albumin in the blood, forms an adduct leading to DNA damage. The epoxide preferentially binds to mitochondrial DNA resulting in hepatocarcinogenesis. When AFB1 bind to DNA at guanine site in liver cells, it can affect the genetic code of enzymes which regulate cell growth. This results in formation of tumors. Aflatoxins are known to bind and interfere with enzymes and substrates that are needed in the process of initiation, transcription and translation in protein synthesis by forming adducts with DNA, RNA and proteins(Sarma *et al*., 2017). In general, it can cause Apoptosis result Cell death, inhibits of nucleic acid (DNA – RNA) synthesis which cause Mutation and Cancer, decrease protein synthesis and Stunting the of growth children, affects membrane stability and lead to cell damage(Min *et al*., 2021).

2.8. Clinical sign of aflatoxicosis

2.8.1. Aflatoxins in animals

Animal can get aflatoxin through contaminated feed. The disease can be acute or chronic depending on dosage and frequency of exposure. Sensitivity varied with species, age and sex of animals. Young species were most susceptible compared to older. Acute toxicity cause death of the animal while chronic toxicity caused loss of appetite, reduction in growth, congestion of liver and hemorrhage, muscular spasms, nervousness, depression in total RBC and WBC counts, reduced hemoglobin content, reduce milk and egg production, recurrent infection as a result of immunity suppression and gastrointestinal hemorrhages and disorder of digestive system. Aflatoxin B1, M1, and G1 are known to cause different types of cancers in different animal species. AFB1 is identified by the International Agency for Research on Cancer (IARC) with sufficient evidence as class I carcinogen (Sarma *et al*., 2017).

2.8.2. Aflatoxins on humans

Humans are exposed to aflatoxins could be either through consumption of contaminated food directly (AFB1) or through animal origin food, especially milk. Milk contamination with AFs is a big problem since milk is an essential nutriment to keep humans healthy and strong specially for children and it is a rich source of calcium, phosphorus, essential amino acids and vitamin due this children are more exposed (Naeimipour *et al*., 2018).

The clinical sign observed in human are:- Abdominal pain, vomiting, pulmonary edema, convulsions, coma, liver damage and the symptoms of acute aflatoxicosis are oedema, haemorrhagic necrosis of the liver and profound lethargy, while the chronic effects are immune suppression, growth retardation, cancer and even death(Kumar *et al*., 2017). Chronic case has teratogenic effect associated with congenital malformation. Aflatoxins are also having mutagenic and carcinogenic effect. Mutagenic effect leads to mutation in genetic code, alteration in DNA which lead to chromosomal breaks, rearrangements, loss or gain of chromosome or changes within a gene (Abrar *et al*., 2013;Sarma *et al*., 2017).

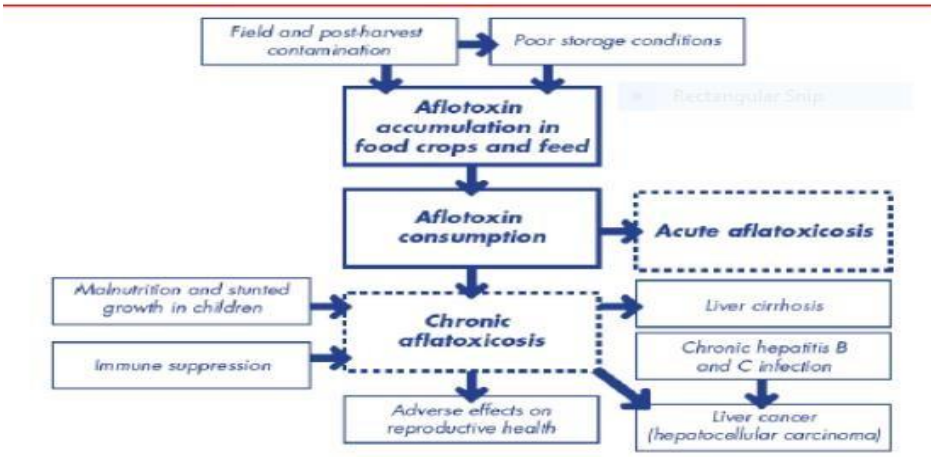

Figure 4. Aflatoxin disease pathways in human source (Seid and Mama, 2019).

2.9. Diagnosis technique of aflatoxin

Diagnosis of aflatoxin is difficult due to variation in clinical sign and presence of secondary infection since it is immuno suppressive. But sign like vomiting, abdominal pain and hemorrhaging, pulmonary edema, acute liver damage, loss of digestive tract function, convulsions, cerebral edema, and coma can also help. History of feed of animal and laboratory detection of the toxin is widely used on today's world. Detection and quantification of aflatoxin in food and feed of animal is a very important aspect of food safety concerns. Aflatoxins can be detected and identified depend on their absorption and emission spectra (Kumar *et al*., 2017).

Mostly used methods are rapid screening methods like Enzyme-linked immunosorbent assays (ELISAs), Immunochemical assays involving detection by electrochemiluminescence (ECL-IA), ELISA using fluorometric detection and, more recently, biosensor assays can be used. Quantitative methods such us Gas chromatography (GC), liquid chromatographic (LC), Thin layer chromatography (TLC), and high-performance liquid chromatography (HPLC) (Ketney *et al*., 2017;Vaz *et al*., 2020).

2.9.1. Enzyme-linked immunosorbent assays ELISA

This method is based on an antibody–antigen (Ab–Ag) reaction and use specific antibodies to detect immunogens, which contain the targeted chemical structures. (Vaz *et al*., 2020). The procedure is, toxin extracts are mixed with enzyme-conjugates and the mixture is placed in wells of antibody-coated microtiter plate. Contaminating sample and enzyme-conjugated compete for binding sites on the antibody. Excess unbound toxin and enzyme-conjugated are washed away. The enzyme substrate is then added to each well and a reaction catalyzed by bound enzyme results in a coloured product that can be then measured. Intensity of colour depends on amount of enzyme-conjugated toxin bound to anti- bodies present. Colour change can be evaluated visually or by measuring absorbance. ELISA shows some disadvantages, such as long incubation periods and several washing and mixing stages (Ketney *et al*., 2017).

2.9.2. Electrochemical biosensors

The detection principle is the binding of the analyte of interest to the complementary bio- recognition element immobilized on a suitable support. When the analyte binds the element, a specific interaction takes place, which results in a change of several physicochemical properties like pH and the transfer of electrons, mass or heat, which are detected with the aid of a transducer. Depending on the transducing method of the signal, the biosensors can be electrochemical, optical, electric or piezomagnetic. In the case of aflatoxins detection, the most frequently used biosensors are electrochemical and optical ones (Ketney *et al*., 2017).

2.9.3. Chromatography methods

Chromatography analysis is based on distribution or partition of a sample solute between stationary phase and mobile phase. Chromatographic techniques can discriminate between a large number of substances analyzed, including those with different chemical structures(Rahmani *et al*., 2009). The general procedure of all chromatography methods includes sampling, sample preparation, extraction, purification and concentration of the extract obtained before the separation, quantitation and confirmation steps(Jaimez *et al*., 2000). Gas chromatography (GC), liquid chromatography (LC), High performance liquid chromatography (HPLC) and thin layer chromatography (TLC) are the widely used techniques.

2.9.4. GC–MS technique

Gas chromatography (GC) was introduced in the field of mycotoxins in the early 1970s. When mycotoxins are sufficiently volatile at the column temperature or can be converted into volatile derivatives, GC can be applied for their determination (Rahmani *et al*., 2009).

2.9.5. Thin-layer chromatography (TLC)

TLC is a standard AOAC method for aflatoxin analysis since 1990. It is widely used for the quantitative analysis and quality control of food products. TLC has been used to determine mycotoxins for a long time. The toxins are extracted from the samples and spotted on silica TLC plates and the toxins separated by running the plate in a range of organic solvent mixtures as mobile phase. The separated toxins can then be visualized by observing under long wavelength UV light (365 nm) or by spraying with specific chemicals to make the toxins spots visible. The toxins can then be quantified by visual or densitometric comparison with standards(Gnonlonfin *et al*., 2013).

2.9.6. Liquid chromatography

LC is a recent and advance method. It can provide good sensitivity, high dynamic range and versatility. It includes both normal and reverse-phase separations. However, most current methods use reverse-phase HPLC, with mixtures of methanol, water and acetonitrile as mobile phases (Vaz *et al*., 2020). The extract can be derivatized with trifluoroacetic acid (TFA) and analyzed by reverse-phase HPLC. The toxins can be detected using either UV or fluorescence detectors or mass spectrometry detection with precolumn derivatization or post column derivatization(Ketney *et al*., 2017).

2.9.7. High-performance liquid chromatography

HPLC is one of the most frequently used methods for detecting and quantifying aflatoxins in food products. It is one of quantitative methods that is suited for online cleanup of sample

extract and could be combined with different detectors. It uses fluorescence detection and a post column derivatization agent such as pyridinium hydrobromide. The polar nature of mycotoxins and their solubility in water and organic solvents such as methanol and acetonitrile suggest that they are amenable to separation on reverse-phase HPLC columns and this has resulted in a diverse array of methods. HPLC is suited for mycotoxin separation and as can be gauged from the compilation of a database of retention times, retention indices, UV absorption is widely used. HPLC detection has mostly been achieved with UV and fluorescence detectors. Aflatoxin's mixtures can be separated on normal phase silica columns using solvent mixtures consisting of chloroform, acetonitrile, cyclohexane, and ethanol or reverse-phase column with C18 packing material (Rahmani *et al.*, 2009).

HPLC have more advantage than thin layer chromatography and enzyme-linked immunosorbent assay in speed, automation, improved accuracy and precision(Sani and Nikpooyan, 2013). The disadvantage of chromatography is it require qualified operators, extensive pretreating of samples and expensive equipment(Ketney *et al*., 2017).

2.10. Public health significance of aflatoxin

Exposure of human to can be by consuming contaminated animal product such us milk, meat and egg or by direct to contaminated crops. Aflatoxicosis is both a food safety and public health issue (Ogodo and Ugbogu, 2016). Milk and milk products are highly nutritious foods containing macronutrients, vitamins, and minerals that are essential for growth in humans especially for children. However, milk and milk products may contain aflatoxins (AFM1) that cause health risks to humans(Ayelign and Saeger, 2020). Aflatoxin B1 has toxic effects both on animals and human. They are potent hepatotoxic, immunosuppressant, and mutagens and carcinogens. aflatoxin is heat stable and cause effect in low concentrations due to their high toxicity(Ponzilacqua *et al.*, 2018).

Exposure to AFM1 can be acute or chronic aflatoxicosis, based on the duration and amount of exposure. Aflatoxin can exert toxicity by altering intestinal integrity or decrease the expression of cytokines which can lead to stunted growth in children and/or

immune suppression (Sarma *et al*., 2017). Specific P450 enzymes in the liver can metabolize/convert aflatoxin into a reactive oxygen species (aflatoxin-8,9-epoxide), this can bind to proteins and cause acute toxicity (aflatoxicosis) or it can be bind to DNA and cause liver cancer (Wu *et al*., 2011). Chronic expo- sure to AFs may lead to impaired immunity, reduced uptake of nutrients from the diet, and growth retardation(Farombi *et al*., 2009). Its high concentrations are lethal, medial concentrations are chronic poisoning and continuous exposure to low concentration of the toxin can result hepatic cancer. Aflatoxin M1 is heat treatment resistant and there is a probability of poisoning by this toxin when consuming infected pasteurized milk. However, Tumorigenesis and mutagenesis (cause birth defect in children) of aflatoxin M1 is less than aflatoxin B1(Creppy, 2002).

Acute Aflatoxicosis in humans has been reported worldwide especially in countries like Taiwan, Uganda, India, Kenya and many others(Seid and Mama, 2019).

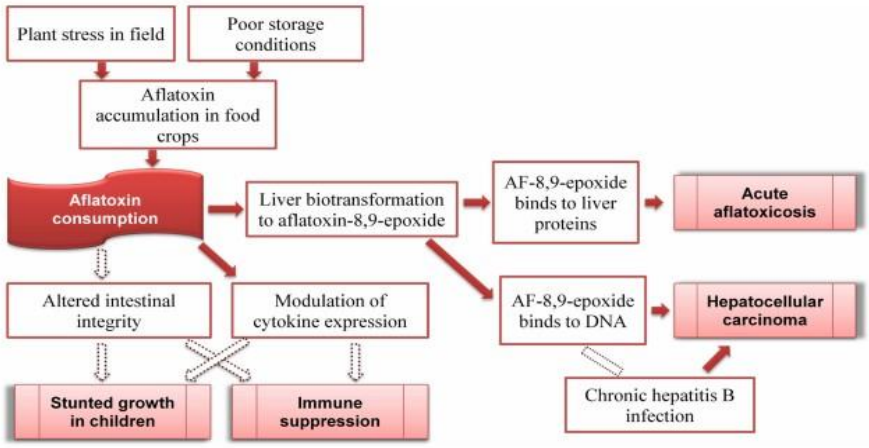

Figure 5. Aflatoxin disease pathway and its effect

2.11. Status of aflatoxin M1 in Ethiopia

In Ethiopia, the status of level of aflatoxin in milk, its level of contamination in animal feed and its veterinary and public health hazard has not been well studied at a national level. Few studies carried out so far showed clearly the significance of aflatoxin contamination particularly in animal feed and milk products. Study in Addis Ababa milk shed between September 2014 and February 2015 shows aflatoxin is almost prevalent in all feed and milk sample (Gizachew *et al*., 2016). The result shows only nine (8.2%) out of 110 sample contain less than or equal to 0.05ug/l, other 101(91.8%) sample contain above the limit of EU standard and about 29 sample (26.3%) contain above 0.5ug/l of USA standard. 26.2% from feed sample contain above 100ug/kg and they determine noug seed cake as main cause of aflatoxin in milk. Other study by(Fikere, 2017) on dairy feed around Addis Ababa indicated high prevalence of aflatoxin (58.3%) in noug seed cake. Report of study by (Besufekad *et al*., 2018). from Gurage zone shows high prevalence of aflatoxinM1(68%) in milk samples.

Risk of human exposure to AFM1 contaminated milk is a major concern in Ethiopia where dairy farmers commonly use different mixed concentrate feeds containing traditional brewery by- product ("atela"), wheat bran, noug (Guizotia abyssinica) cake, maize grains, and silage to increase milk production, where these feeds are susceptible to AFB1 contamination(Chauhan *et al.*, 2016). Other study by(Mesfin *et al*., 2018). From central high land of Ethiopia, shows controversial result of zero prevalence or none of the sample contain above permissible level of EU standard of (0-0.05 and USA standards of (0.5 μg/L). Study by (Tadesse *et al*., 2020). Showed the higher level of AFM1 contamination **52%** in almost half of the analyzed milk samples from Bishoftu (Ethiopia). Study by (Eshete *et al*., 2020) in southern Ethiopia indicated that, foods intended for infants are heavily contaminated with AFB1(96.4%) and they report the highest contamination of breast milk during dry season, which is 64.4% of the sample was contaminated with AFM1. This all shows the distribution of AFM1 level has increased and requires serious considerations.

2.12. Prevention and control methods of aflatoxin

Control of aflatoxin is both for public health importance and economic improvement. Except for supportive therapy (e.g., diet and hydration) there are almost no treatments for aflatoxin

exposure. Methods of controlling aflatoxin exposure are largely prophylactic. Once it is produced, they are stable in many foods. Common cooking methods such as boiling, pressure cooking (autoclaving) and pasteurization will not be able to remove aflatoxins from milk completely. Boiling can only reduce about 50%-70% so, Aflatoxin are also found in posturized milk(Taherabadi *et al*., 2016). So control at source is the most effective way(Bennett *et al*., 2007).

Good hygienic practice during harvesting, drying, storage and processing of agricultural products, Continuous surveillance and monitoring, awareness creation, using different detoxifying and absorbent (like probiotics, activated carbon and chemicals like H2O2) agents are very important prevention methods (Augusto *et al.,* 2013; Mesfin *et al*., 2018). Feeding ration free feed for dairy cow is major way of prevention AFs in milk (Gnonlonfin *et al*., 2013). Some studies showed that vaccination might reduce the risk of contamination of animal/dairy products by AFs. The levels of anti-AFB-1 antibodies in vaccinated heifers decrease during pregnancy and after calving and at the beginning of the milk production cycle, they back to the previous range. Such changes in levels of anti-AFB-1 antibodies after vaccination may show as the effectiveness of the vaccine. Moreover, temperature and moisture of feed during storages should be controlled since they can induce the fungal growth (Naeimipour *et al.,* 2018).

3. MATERIAL AND METHODS

3.1. Study area description

The study was conducted in major Addis Ababa milkshed area (Sululta and Sebata). These study areas were selected because they are among the major milk supplier sites to Addis Ababa both for household raw milk consumption and for milk processing plants.

Sebata is located in special zone of Oromia regional state in central highlands of Ethiopia at 24 km west of Addis Ababa on the main road to Jimma. The average annual rainfall is 1100 mm, more than 85% of which falls in the main rainy season (June to September). The altitude of the area ranges from 2200-2600m asl and the average annual temperature range from 6-21^{O}C (Desalegn, 2018). According to Sebeta-Awas district's livestock agency there is both intensive and semi-intensive farming system and there are about 310 dairy farms in the study area. Daily milk production of the area were about 20,000 liters, which only half is marketed to Addis Ababa through formal market, the rest is either consumed at household level or processed as traditional dairy products(Brandsma *et al*., 2012).

Sululta district is located between 9° 13′–10° 57′N latitude and 37° 57′–39° 33′E longitude. It is 40 km North of Addis Ababa at an average altitude of 2,550 m asl. The annual rainfall is minimum of 834 mm and a maximum of 1,447 mm. The mean minimum and maximum temperatures of the area are 4.4 and 22.5 ℃, respectively(Beyecha *et al*., 2012). According to Sululta District Livestock Production, Marketing and Health Agency office, the total cattle population of the district for the year 2011 was 210,211 heads. Both intensive and semi-intensive farming system is practiced in the study area and there are about 500 dairy farms. From 2010 to 2011 the average annual milk production of the area is 6,694,750litres and average daily milk production is 21,950liters, from this about 16,462 liters is marketed formally and about 5,488liter is retained at home (Brandsma *et al.*, 2012). Above 6 milk processing plant receive raw milk from both study areas.

3.2. Study design and sample size determination

A cross-sectional study design was used to conduct the study on determination of aflatoxin in raw and pasteurized milk in central Ethiopia from October 2020 to May 2021. The desired sample size was calculated by using the formula given by Thrusfield (2005) with 95% confidence interval and 5% precision and 91.8% expected prevalence based on previous study by ILRI (Gizachew *et al*., 2016).

$$n=\frac{Z^2\ Pexp\ (1-Pexp)}{d^2}$$

Where, n= required sample size

Z=statistic for level of confidence at 95CI which is 1.96

d= desired absolute precision or margin of error = 0.05

Pexp=expected prevalence which is 91.8%

$$\mathbf{n} = \frac{(1.96)^2\ [0.918(1-0.918)]}{(0.05)^2}$$

n =113

For this study 114 sample were collected based on previous prevalence by simple random (60 raw milk from farm and 54 pasteurized milks from different brands). Simple random sampling was used to select the farm in the districts and sample size for each district was proportionally allocated.

3.3. Sample collection

About half liter (500ml) pooled raw milk was collected from 60 different dairy farms. The sample was taken after the milk was mixed well in container. This help to homogenous the milk and to take appropriate sample. For the pasteurized milk, 54 packs of 500ml were purchased from supermarket from three different brands and transported (at a temperature of +4^{O}C in ice box) to VDFCA laboratory as soon as possible and stored at -20 ^{O}C until analyzed. For each farm owner there was questionnaire/interview on type of feed they used,

their feed management practice (storage place, time and way of storage), source of feed and their knowledge on aflatoxin.

3.4. Sample preparation and laboratory investigation

3.4.1. Sample preparation procedures

The extraction and clean up procedure of sample preparation were based on AOAC 2002 official method. Frozen milk samples of 100ml were thawed using a water bath at 40°C for 30 minutes. After heating and bringing it to room temperature centrifuge it at 4500rpm for 15min. This help to separate the fat from the milk and to remove easily by using spoon and then filtered by using syringe filters through Whatman No.4 filter paper and transferred into a 50 mL tube. Then 50 mL of defatted (skim) milk was completely passed through Afla M1^{TM} Immunoaffinity Column (Afla CLEAN produced by LCTech GmbH company of Germany) at a rate of about 1-2 drops/second and let all of the sample to drain through the column until there is no more sample in the column, at this time antigen antibody bond were formed. Then the column was washed with 10 ml of distilled water at a rate of 1-2 drops/second. Residual water was removed by gentle gas stream or vacuum. Then 3ml of acetonitrile was added and wait for 5min to break the analyte- antibody bond. After 5 min the column were open and transfer into 10ml centrifuge tube and evaporated/concentrated under nitrogen stream. Finally, the sample were reconstituted by 1ml of the mobile phase solution of Water-Acetonitrile-Methanol (60:25:15) and transferred to amber glass vial and ready for HPLC detection (2000.08 AOAC official method, 2002).

3.4.2. HPLC conditioning and injection procedures

HPLC machine was conditioned through pumping mobile phase solution of Water-Acetonitrile-Methanol (60:25:15) at steady flow rate till stable baseline develop. Working standard solutions were prepared at concentrations of 0.05, 0.1, 0.5, 1, 1.25, 1.5, 2 and 4 AFM1 μg/L in mobile phase to construct the calibration curve. The optimal conditions of

instrument were checked with aflatoxin M1 calibrant solution before analyzing test sample. Then, linearity of injection of calibrant solutions and stability of chromatographic system were checked and Fixed amount of aflatoxin M1 calibrant solution were repeatedly injected until stable peak areas was obtained. Peak areas corresponding to consecutive injections were within ± 5 %. After HPLC output the calibration graph was prepared by plotting the peak area against the mass of injected aflatoxin M1. By using the same conditions as for the calibrant solutions, the test sample were injected according to stipulated injection scheme/by ordered sequence or single injection(2000.08 AOAC official method, 2002).

Once, aflatoxin M1 peak area is determined, aflatoxin M1 concentration in test sample were calculated from the calibration graph in ug/l. The formula used to calculate AFM1 were: Wm = Wa x (Vf/Vi) x(1/Vs). Where, Wm= the numerical value of aflatoxin M1 in test sample in ng/ml or ug/l. Wa= the numerical value of the amount of aflatoxin M1 corresponding to area or height of the aflatoxin M1 peak of the test extract(ng). Vf= the numerical value of the final volume of redissolved elute(uL). Vi= the numerical value of the volume of injected elute(uL). Vs= the numerical value of the volume of prepared test portion passing through the column(ml).

The HPLC system were interfaced, via network chromatographic software (Agilent Chem Station), to a personal computer for instrumentation control, data acquisition and processing (2000.08 AOAC official method, 2002).The result was interpreted according to Ethiopian standard agency regulatory limit which is 0.05ug/l in raw/liquid milk.

3.5. Questionnaire survey

A questionnaire survey was used to assess the potential risk factors associated with contamination level of AFM1 at individual farm level. The questionnaire was prepared by targeting farm owners and the questions were concerned on the major risk factors of Aflatoxin like storage time of the feed, moisture content of the feed, ventilation of feed storing room, type of commonly used feed, quality of the feed and the knowledge of farmers on aflatoxin. All necessary information was gathered through this structured questionnaire and also by indirect observation to the farm.

3.6.Ethical clearance

Ethical clearance was obtained from animal research ethical review committee of Addis Ababa University College of Veterinary Medicine (Certificate Ref. No: VM/ERC/28/06/13/2021, Date: 28/03/2021).

3.7. Data management and analysis

All collected data was organized, coded and entered to Excel spread sheet (Microsoft® office excel 2016) and exported to R-statistical software (version R-3.5.1) for appropriate analysis. Linear logistic regression was used to study the association between AFM1 contamination level with the considered risk factors individually and for variable with small positive or negative result fisher exact test was used. The magnitude of the risk factors for the occurrence of aflatoxin were compared by using an odds ratio. Descriptive statistics (Maximum, minimum, mean, SD) was used to present the result of AFM1 contamination level of milk source and milk type. Table of frequency was used to figure out the finding of the questionnaire.

4. RESULTS

The result of calibrant solution shows equation of regression as y=2.5401x +0.0073, where y= peak area and x= amount of AFM1. Linearity (r) of concentration with their respective peak area was 0.99937 and the coefficient of determination (R) was 0.99875. The retention time of AFM1 was 4.6minute.

The HPLC analytical result showed high contamination of milk samples. From total of 114 analyzed milk sample 29(25.4%) of them have AFM1 of above permissible level of the European community and Codex Alimentarius recommended limit which is 0.05 µg/L in liquid milk. The minimum AFM1 detected was 0 and the maximum was 0.893 With mean and SD of 0.0465 and 0.102 respectively. From 114 sample 79(69.3%) of them was contaminated by AFM1 or they contain detectable amount of aflatoxin M1. Only 2 samples (1.8%) of 114 analyzed milk samples exceed the maximum limit set by US which is 0.5 µg/L in liquid milk.

4.1. Raw milk analysis

From total 60 raw milk 10 (16.7%) of them have AFM1 above permissible level of 0.05 µg/L. The mean and SD were 0.0469 and 0.1367 respectively. About 50% of analyzed raw milk was positive or have detectable amount of aflatoxin M1.

Table 1. Descriptive statistics of AFM1 Level in raw milk in the study area

Factors	No sample	positive	Above 0.05 µg/L	min	max	Mean ±SD
Location Sebata	30	20	8	0	0.893	0.082±0.184
Sululta	30	10	2	0	0.2018	0.012±0.038

Sample from Sebata distinict was more contaminated, where 66.7% positive and 26.7% of the sample was contain above permissible level than those from Sululta distinict, 33.3% of the sample were positive and 6.7% of them were contain above permissible level. Considered risk factors (source of sample collection, feed source, presence of noug seed cake in feed, type of milk and storage time of feed) have statistically significant difference.

Table 2. Contamination level of AFM1 with considered risk factors

Variables		**No of sample**	**Positive**	**Above 0.05 µg/L**	**P_value**
Location	Sebata	30	20	8	
	Sululta	30	10	2	0.08
Feed source	graze	37	11	2	
Not use grazing		23	19	8	0.005
Industry byproduct					
With noug seedcake		26	19	9	1.667e-06
Without noug SC		34	11	1	
Storage time	1week	37	8	1	
Above1week		23	22	9	1.204e-06
Type of milk	Raw	60	30	10	
Pasteurized		54	52	19	0.026

4.2. Pasteurized milk analysis

A total of 54 pasteurized milk was analyzed from different brand. 19(35.2%) of them have above permissible level of 0.05 µg/L. The minimum AFM1 detected was 0 and the maximum was 0.119 with mean and SD of 0.046 and 0.037 respectively. The toxin (AFM1) was detected in almost all of them (52/54 or 96% of them).

The test statistics showed that there was no statistically significance difference ($P>0.05$) between different brand of pasteurized milk. Which means AFM1 was found in all brand of

pasteurized milk which was included in this study. This can approve the heat resistant properties of AFM1 which stated in different study and have significant public health effect to human being. The following table show the result AFM1 in pasteurized milk from different brand.

Table 3. Contamination Level of AFM1 in pasteurized milk by their respective brands

Factors		No sample	Positive	Above 0.05ug/L	Mean	SD	P value
Brand	**1**	13	12	6	0.052	0.032	0.782
	2	20	26	6	0.038	0.037	0.148
	3	15	14	7	0.055	0.041	0.978

4.3. Comparing AFM1 contamination level in raw and pasteurized milk

There was statistically significant difference between raw and pasteurized milk contamination by aflatoxin M1(p-value = 0.026). The result of this study indicates that,50% of raw and 96.4% of pasteurized milk were contaminated by AFM1 and 16.7% of raw and 35.2% of pasteurized milk contain AFM1 which is above the maximum limit of 0.05 ug/L.

Table 4. Contamination Level of AFM1 by type of milk (in raw and pasteurized)

Type of milk	No. sample	Positive	Above 0.05 ug/L	Min	max	p-value
Raw	60	30(50%)	10(16.7%)	0	0.893	0.026
Pasteurized	54	52(96.4%)	19(35.2%)	0	0.119	

4.4. Farmer's knowledge and awareness on aflatoxin

Survey data was collected during sample collection from the dairy farm owners to get complete information and to associate the result with their answers. Indirect observation was also done on feed management of the farmers. The result was summarized in Table 5.

Table 5. Knowledge Attitude and Practice (KAP) of dairy farm owners on Aflatoxin contamination.

Knowledge on aflatoxin related	**Response**	**Frequency**	**Percentage %**
Knowledge on mold growth and formation of the toxin	Yes	41	68.3
	No	19	31.6
Knowledge on favorable conditions for mold growth on animal feed	Yes	41	68.33
	No	19	31.66
Do you know or heard of aflatoxin	Yes	6	10
	No	54	90
Do you know that aflatoxin cause disease in animals	Yes	6	10
	No	54	90
Do you know aflatoxin can pass through milk to consumer and have effect on human	Yes	6	10
	No	54	90
Do you think that aflatoxin can be destroyed by pasteurization of the milk	Yes	49	81.7
	No	11	18.3
Knowledge on feed management practice			
How do you store the feed	In house	50	83.3
	In shade	8	13.3
	Open field	2	3.4
Is there ventilator in the feed storing house	Yes	60	100%
	No	0	
do you control moisture content of the feed	Yes	45	75
	No	15	25
do you check quality the feed while buying and feeding	Yes	6	10
	No	54	90

5. DISCUSSION

The present study showed that there is a widespread contamination of milk by aflatoxin in raw and pasteurized milk originated from Sebata and Sululta milkshed areas. From a total of 114 analyzed milk samples, 25.4% of them exceed the maximum permissible level of Ethiopian standard agency and EU level of 0.05 µg/L. This result is lower than the result of previous study by (Gizachew *et al*., 2016) in great Addis Ababa milkshed area in which 91.8% of analyzed samples contained above permissible level of 0.05 µg/L. This difference can be due to attitude change of the farmers specially on feed type used since 2016. Most of the farmers reduce the amount of noug seed cake in their dairy feed and also reduce the time of storage of the feed which is purchased from feed retailers. Study from Injibara report low contamination level AFM1 with 10% of analyzed sample have above permissible level and 15% of the sample was contaminated by AFM1 (Kassa1 *et al.,* 2020). This may be due to difference in environmental condition or climate change, type of feed used and feed management practice of the farmers in Injibara.

The current study showed that 79(69.3%) of 114 analyzed sample were contaminated by AFM1. This result was much lower than the study from Bishoftu by(Tadesse *et al*., 2020) who report that, from 108 analyzed milk samples, all samples(100%) were found to be contaminated by AFM1 with a mean value of 0.835 µg/l. The result of this study was in line with study from Kenya by (Anyango *et al*., 2018) who report 26.4% of analyzed sample was exceed the limit of EU. Study from Pakistan by (Ahmad *et al*., 2018) report that 93% of the analyzed samples were contaminated by AFM1 and 69% of the samples exceeded the EU ML (0.05 µg/L) which is much higher than the result of this study.

In present study, in raw milk AFM1 was detected in 50% of them and from those positive samples 16.7% (10) of them has above permissible level of 0.05 µg/L. This is lower than study from Kenya by (Kagera *et al.,* 2018) who report that,99% of 84 analyzed milk sample were contaminated by AFM1 and 64% were exceed the permissible level of EU 0.05 µg/L The result of this study were also much lower than study report of (Asghar *et al*., 2018), who report 91.7% contamination and 80.1% above permissible level from 156 tested samples of raw milk. The

differences shown between this study and others might be related to the difference in geographic location, feed management system and climatic conditions of the area which might associated with the growth of the fungus.

In comparing the contamination level of raw milk by aflatoxin by location of sample collection, raw milk collected from Sebata district has high contamination level of AFM1(26.7%) than sululta district (2/30 or 6.7%). This can be due to environmental temperature (high temperature in Sebata), feed type used and farming system. Most of farmers in Sebata district don't have free grazing land for their dairy cattle so that they use purchased feed under intensive farming system but farmers from Sululta district use semi-intensive system and they use grazing on their backyard around home and crop byproduct during harvesting of crops. This can help to reduce exposure of their dairy cows to aflatoxin originated feed.

In the present study, contamination level of AFM1 was higher in the pasteurized milk than that of raw milk. Because the processing plants can collect from different farm and mix it together this can increase the chance of contamination. But for the raw milk it was at farm/individual level so the contamination level was low. This result is in line with study done by(Abyaneh *et al*., 2019) who report high contamination in pasteurized milk and state that contaminated bulk milk is often mixed with uncontaminated milk in dairy plants, as these sometimes have to combine milk from several sources to achieve the necessary production volume and this can increase chance of contamination. Study by (Taherabadi *et al.,* 2016) showed that 9.27% AFM1 contamination in raw and 5% in pasteurized milk, which showed lower level of contamination than the present study.

The present study revealed that high AFM1 contamination was found in pasteurized milk than raw milk. But the maximum AFM1 concentration was found in raw milk. This result was in line with study in Iran in 2017 by(Abyaneh *et al*., 2019) ,who reported high AFM1 contamination in pasteurized milk than raw milk, but they found high concentration level of AFM1 in heat treated milk. Report by(Sharma *et al.,* 2019) showed that high contamination level of AFM1 was found in pasteurized milk and they try to indicate thermo stability of AFM1 which is in line with this study. Study by(Sani and Nikpooyan, 2013) report low contamination level of

AFM1 in pasteurized milk, But all sample was positive for AFM1. Study report from Iran by (Taherabadi *et al., 2016)* indicate low contamination in pasteurized milk (5%) than raw milk (9.2%) which were not in line with the result of the present study.

The present study showed that considered risk factor like storage time, feed type and grazing have significant effect on aflatoxin production. Storage time of feed have significant effect on mold growth and aflatoxin production. Feed which was stored for long time (above one week) have more AFM1 than feed stored less than one week. But the toxin was detected in both storage time. This can due the moisture content of the feed, environmental climate condition, ventilation and other factors. This result was also supported by the study done by (Abyaneh *et al*., 2019) who reported storage condition of feed can increase chance of mold growth.

Feed type have significant effect for aflatoxin production. Some feed can easily be contaminated by AFB1 which can be transformed to AFM1 in lactating cows. In this study milk from farmers who fed their lactating cow with feed which contain noug seed cake have high contamination level of AFM1 than other feed type like brewery grain and wheat bran. This is in agreement with the previous study by (Gizachew *et al.*, 2016) who report high contamination level in feed which contain noug seed cake.

Grazing has also significant effect on contamination level of AFM1. In this study high contamination level was recorded in milk originated from farmers who didn't use grazing and low AFM1 contamination in milk originated from farmers who practice open grazing system at backyard or in grazing field and in addition to concentrate feeds for their dairy cows.

With regards to the result from KAP survey, from 60 interviewed dairy farm owners 15(%) were females and 45(%) were males. Their educational back ground was 6.7% farmers was illiterate, 41.7% were attend primary school, 45% attend secondary school and 6.6% farmers attended higher educations.

All (100%) of dairy farm owners was offering concentrate feed to their lactating cows. Majority (76.7%) of farmers from Sebata district practice zero grazing due to lack of grazing land in the

area and those farmers were obligated to relay on purchased pasture, fodder and concentrates, which increase chance of aflatoxin contamination. According to the survey result 68.3% of the farmers have awareness on favorable condition for mold growth and aflatoxin formation. Only 10% of interviewed farmers heard the word aflatoxin and know that it can cause disease in human through milk from intoxicated cows. 81.7% of the farmers assume that aflatoxin M1 can be destroyed by pasteurization or other heat treatment. This assumption is from the fact that pasteurized milk is safe from bacterial but AFM1 is toxin not living organism.

Feed management practice of the farmers was observed during the sample collection and majority (83.3% of them store their feed in house with ventilation and 75% of them had moisture content of in the stored feed. Only 10% of them can check the quality of feed using simple observation while buying the feed but they did not check it by using laboratory examination, they only check the physical appearance of the feed and try to buy from good feed retailers in the area.

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed that raw milk from Sebata and Sululta district were contaminated by AFM1 with 25.4% of them exceeding the maximum limit of EU. Milk from Sululta district were less contaminated than Sebata district. The study indicates that pasteurized milk was highly contaminated by AFM1 with 35.2% of them exceed the maximum limit of EU. This result suggests that both raw and pasteurized milks are unsafe for human consumption in the study area and it requires strict monitoring by focusing on the considered risk factors. The presence of AFM1 in raw and pasteurized milk indicate that the health of the community is in a great risk specially children who consume milk daily.

Based on the above conclusion, the following recommendations are forwarded:

- Awareness and training should be provided to farmers on feed management practice, especially on feed storage and feed quality.
- Screening and monitoring of AFB1 especially in concentrate feed and AFM1 in milk should be done regularly.
- Feed should be strictly managed on farm, at harvesting, storing, feed producing factories, and during feeding of the dairy cows to reduce the possibility of fungus growth in the feed.
- Training should be given to feed producer and retailers to reduce AFB1 contamination level during production and storage time.
- Pasteurized milk should be monitored and checked frequently since AFM1 was not removed by pasteurization or pasteurization did not grant us safety of the milk.
- Screening should be done to AFM1 in milk processing plant before packing pasteurized milk to reduce human exposure.
- Further study on risk mitigation strategies and bio-detoxification method should be done for both AFB1 and AFM1.

- Ethiopia set maximum limit of 0.05ug/l for raw milk which were adopted directly from EU. It should be revised and better to set limit based on Ethiopian population milk consumption capacity and current information of the aflatoxin.

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8.ANNEXS

Annex 1. Chemicals, reagents and equipment used

For this study chemicals and reagents used was: AFM1standard solution, stock solution, working solution and calibrant standard solution. HPLC grade of Acetonitrile, methanol and Deionized water. Afla M1^{TM} Immunoaffinity columns (AFLA CLEAN produced by LCTech GmbH company of Germany) which contain antibodies against Aflatoxin M1 with capacity of 100ng aflatoxin M1 and recovery of 80%. The mobile phases were prepared on daily bases in isocratic modes (water-acetonitrile-methanol in 60:25:15) and sonicated to mix well and degassed to remove air.

Equipment used during the study are: Measuring cylinder (100ml,50ml), Centrifuge, Syringe filter, Evaporation /concentration system MV5 US-MP5000 Automated concentrator, Syringe barrels which used as reservoir (20ml,50ml capacity), glass beakers, soniccater, vacuum system for immuno assay column, conical glass tube, HPLC, with fluorescence detector (FLD), HPLC columns, Liquid chromatography equipment: - with pump delivering flow rate of 1ml/minute, loop injection system of 20-100ul capacity, fluorescent detection with 360nm excitation and 440 nm emission and recorder, integrator or computer-based processing system (2000.08 AOAC official method, 2002).

Annex 2. Interview Questions

Section I. **Verbal consent for dairy farm owners**

Hello, my name is Sitena Kebede Mohammed. I am from Addis Ababa University College of Veterinary Medicine and Agriculture. Currently I am doing a research on aflatoxin M1 which has great public health significance. I want to ask you a few questions about aflatoxin and your dairy feed management practice. The objective of this study is to assess the feed type used and feed management practice of dairy farm owners in relation to AFM1 contamination level in milk. Since feed dairy feed is the main factors for the occurrence of AFM1 in milk. Your cooperation and willingness will be very important in identifying risk the factors and to develop control and prevention strategies. Your name will not be written in this form. Your participation is voluntary and information that you give will be kept secret. In addition, you are not obliged to answer any question that you don't want to answer. Are you volunteer?

Thank you!!!

Section II. questioners for dairy farm owners

Questionnaire for dairy farm owners

1. **Farm Owner Socio-demographic and general information**

Name of respondent/farm holder_____

Gender A) female B) male

Educational status A) primary B) secondary C) higher education D) illiterate

Contact address_____

Location/site_____

Farm no/code_____

Date of collection_____

No of dairy cow_____

Farm scale A) Small-scale (<10) B) medium (10-50) C) large scale (>50)

2. **Feed related question**

2.1 What is the Source of your dairy feed?

A) Grazing B) purchased feed C) both

2.2 Type of feed commonly used beside grazing?

A) Concentrate B) roughage (teff straw, grass bale) C) both D) other dairy feed _____

2.3 Which type of industrial by products (concentrate feed) do you feed your cow commonly?

A) noug seed cake B) wheat bran C) brewery grain D) mixed C) other _____

2.4 Feed store information

Do you store the feed? A) yes B) no

For how much time do you store it? A) below one week B) above one week

How do you store the feed? A) Raise above the floor B) on the floor C) other_____

Where do you store your feed? A) in open field B) in shade C) In house D) other method

Is there ventilator in feed storing house? A) Yes B) no

do you control moisture content of the stored feed? A) yes B) no

do you check quality the feed while buying and feeding your dairy cows? A) yes B) no

3. Knowledge on Mycotoxin/aflatoxin?

3.1 Do you have any awareness on mold growth on feed? A) yes A) no

3.2 Do you know favorable condition for mold growth in feed? A) yes A) no

3.3 Do you know or heard of aflatoxin? A) yes A) no if yes…

3.4 Do you know that aflatoxin cause disease in animals? A) yes A) no

3.5 Do you know aflatoxin can pass through milk to consumer? A) yes A) no

3.6 Do you know aflatoxin have health effect on consumer? A) yes A) no

3.7 Do you assume that aflatoxin can be destroyed by pasteurization of milk? A) yes B) no

4. Health related question

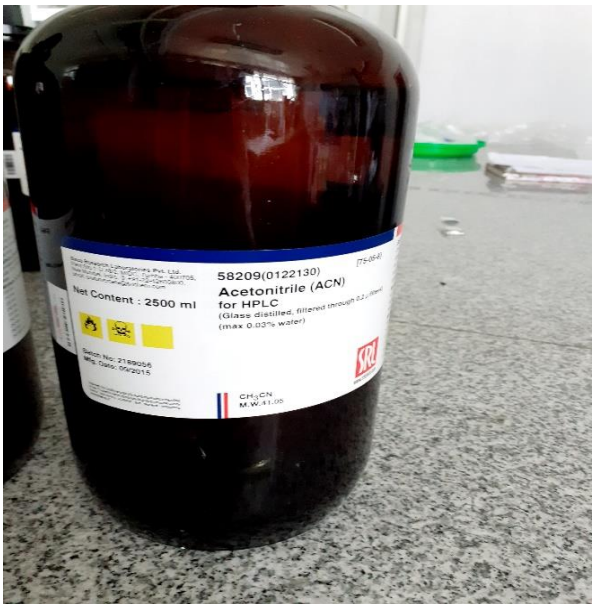
4.1 How do you care health of your animals?

A). By veterinary professions B) self-medication C) by traditional healers

4.2 Do you use medicinal plants? A) Yes B) no

4.3 If yes list them_____, _____, _____.

Annex 3: pictures of laboratory materials used during the study.

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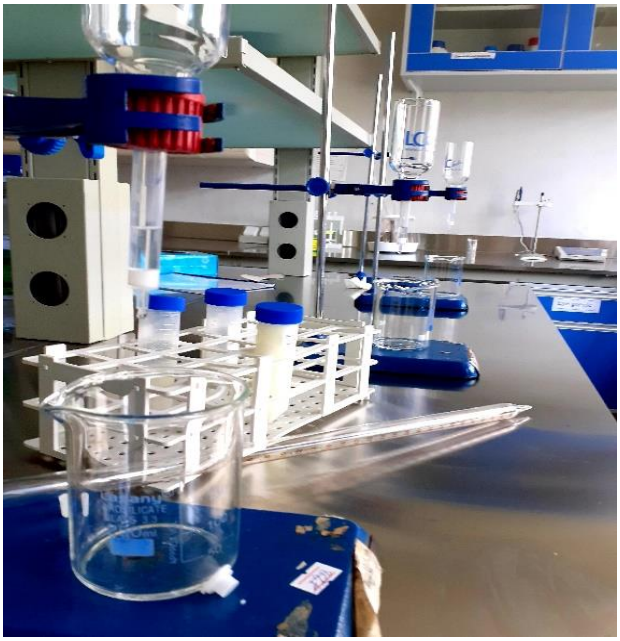
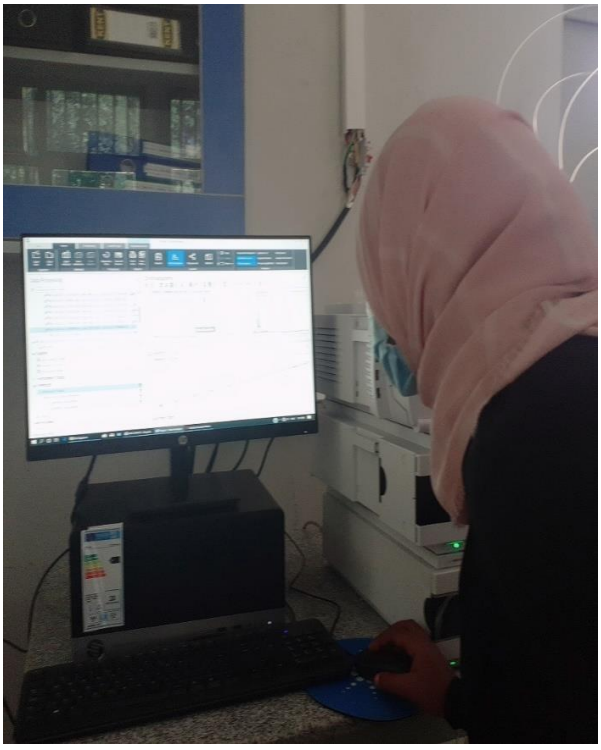
UVE

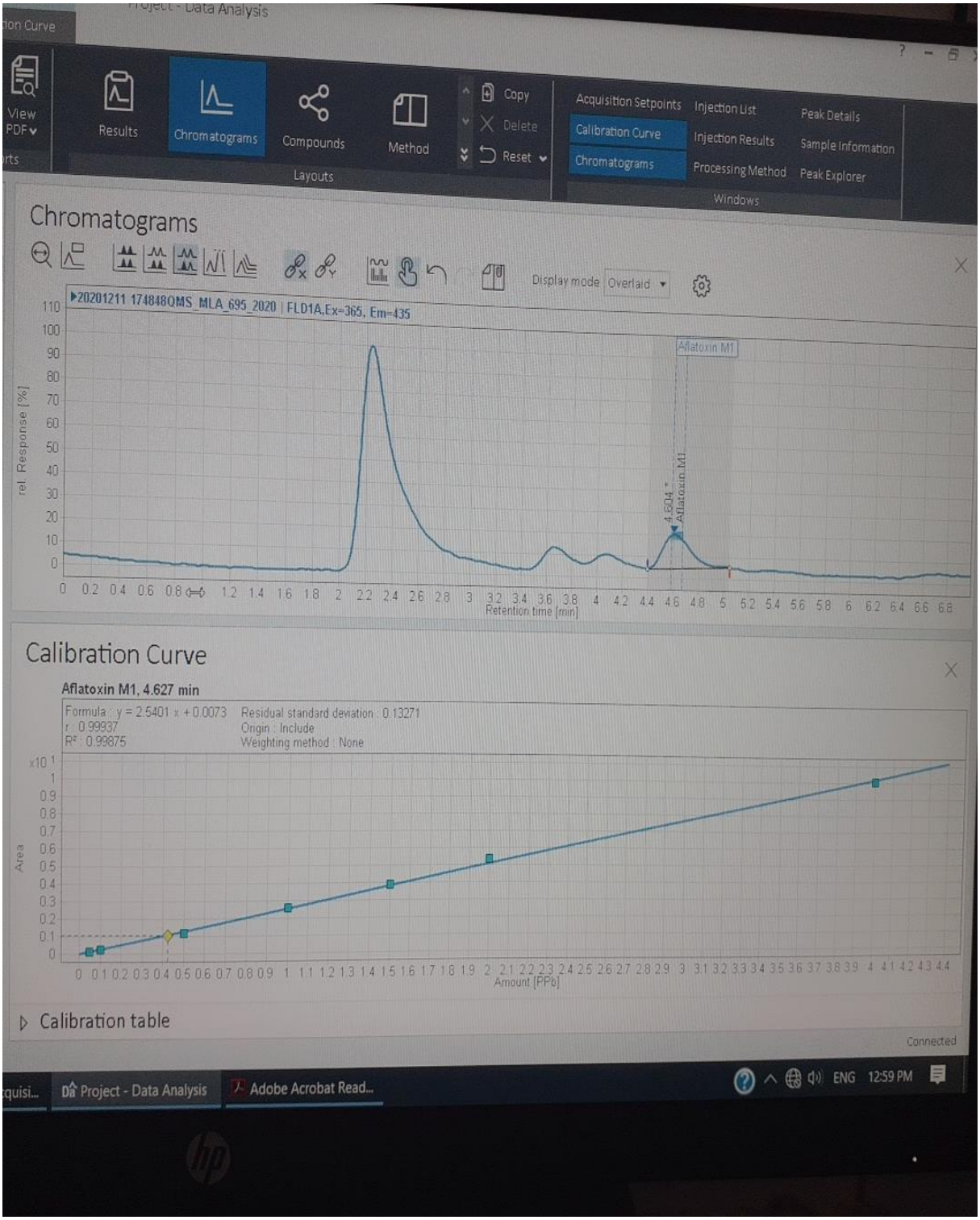
***Annex 4. Pictures taken during sample collections*.**

Commonly used feed in Sebata area

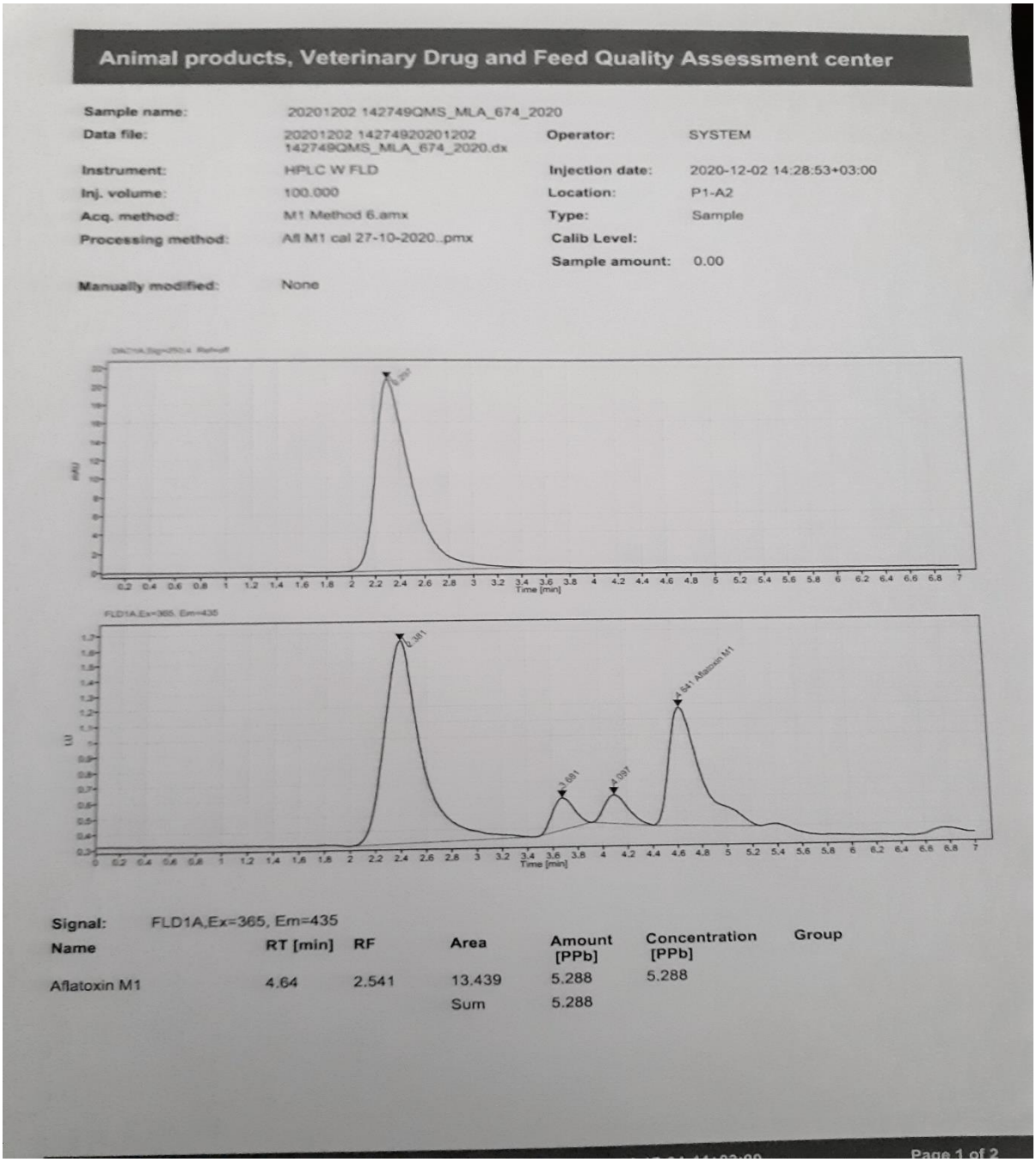
Annex 5. Pictures taken during laboratory work and laboratory results.







Calibration points and their respective peak area



Animal products, Veterinary Drug and Feed Quality Assessment center

Sample name:	20201202 142749QMS_MLA_674_2020		
Data file:	20201202 14274920201202 142749QMS_MLA_674_2020.dx	**Operator:**	SYSTEM
Instrument:	HPLC W FLD	**Injection date:**	2020-12-02 14:28:53+03:00
Inj. volume:	100.000	**Location:**	P1-A2
Acq. method:	M1 Method 6.amx	**Type:**	Sample
Processing method:	All M1 cal 27-10-2020..pmx	**Calib Level:**	
		Sample amount:	0.00
Manually modified:	None		

Signal: FLD1A,Ex=365, Em=435

Name	RT [min]	RF	Area	Amount [PPb]	Concentration [PPb]	Group
Aflatoxin M1	4.64	2.541	13.439	5.288	5.288	
			Sum	5.288		

Page 1 of 2

HPLC result with AFM1 concentration, retention time, peak area and other important point.

Ethical clearance

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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/28/06/13/2021

Name of Applicant: **Sitena kebede (BVSc, MSc fellow)**

Address: Department of Microbiology, Immunology & Vet. Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Determination of aflatoxin in raw and pasteurized milk using high performance liquid chromatography in Central Ethiopia*

Date of application: **December, 2020**

Nature of the project:	**Non-invasive**
Target animal species:	**No animals used: only bulk milk**
Number of animals involved:	**60 dairy farms**
Study area:	**Central Ethiopia**

Minutes No. and date of review: **VM/ERC/06/13/021, 28/03/2021**

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

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary

Getachew Terefe (DVM, PhD, Professor of Vet. Parasitology)
Chairman

Signature

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

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Please quote Our Ref. No. When replying

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