**Sudan University of Science and Technology**

**Collage of Graduate Studies**

# Screening for Antibiotic Residues in Milk of Cattle and Sheep in Khartoum and AL-Gezira States

# الكشف عن بقايا المضادات الحيوية في حليب الأبقار والأغنام في ولايتي الخرطوم والجزيرة

**By**

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## DECLARATION

I,Yousra Mohammmedelhassan, hereby declare to the Sudan university of science and technology that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other Institution.

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***Dedication***

*This work is humbly dedicated to my family for their patience, financial and spiritual support during the period of my study.*

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## List of abbreviations

| Abbreviation | Descriptive meaning |
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| | |
|---|---|
| TPS | Traditional production system |
| MPSDFS | Modern production system for dairy farms |
| HTST | High temperature short time |
| UHT | Ultra high temperature |
| HHST | High heat short time |
| WMP | Whole milk powder |
| PABA | Para amino bezoic acid |
| I/V | Intravenous |
| I/M | Intramuscular |
| S/C | Subcutaneous |
| FDA | Federal drugs administration |
| FOA | Food and agricultural organization |
| WHO | World health organization |
| CVMP | Committee for Medical Products for Veterinary Use |
| EU | European union |
| NOAEL | No observed adverse effect level |
| UEMOA | Union e'conomique at mone'taire ouest africaine |
| VRI | Veterinary Research Institute |

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## ABSTRACT

This study was conducted in Sudan between November, 2017 and April, 2018 to assess the antibiotic residues in raw milk produced by small-scale dairy farms and local markets selling milks in the area, as these residues may have a negative impact on human's health. Fifty small-scale dairy farmers and sellers were involved.Fifty milk samples were randomly collected in douplicate ;meaning 2 samples from each animal to only one of them ,the Sodium azide was added as a long time sample preservative for future researches. The study involved Aljazeera state and three localities in Khartoum state which are East-nile, Al-khartoum and Omdurman . Laboratory assessment included, screening qualitative test using inhibitory activityand the micro-biological methods in which all Samples were tested for the presence of residues of antibiotics .A strain of bacteria *Bacillus subtilis* was used and cultured in agar mediaand the milk samples were placed on cavities of the agar .A milk sample-impregnated whatman's filter paper was used in another method.

Chi- square test was used for comparison between different localities , between farms and markets samples and between cattle and sheep at 5% probability level ;to determine the percentage of antibiotic residues. Out of the fifty milk sample 3(6%)were positive for antibiotic residues.Two of these positives samples were bought from the markets in Omdurman locality while the third one was obtained from a farm within the same locality. The study Recommends a further screening for residues at the milk collection centers and investigation of the milk production practices among small-scale dairy farmers. This will provide a standing ground for designing appropriate and effective small scale milk production practices which will reduce milk contamination and help to protect the health of consumers in Sudan.

# ملخص الاطروحة

هذه الدراسة تم اجراءها في السوادن بين نوفمبر ,2017 ومايو, 2018 لتقييم بقايا المضادات الحيوية في الحليب الخام المنتج بواسطة مزارع الالبان صغيرة النطاق والاسواق المحلية التي تبيع الحليب في المنطقة,بما ان هذه البقايا قد تترك اثرا سلبيا على صحة الانسان.خمسين من مزارعي الالبان صغيرة النطاق والباعة تم ضمنهم.خمسين عينة حليب جمعت عشوائيا كثنائية,بمعنى عينتين من كل حيوان,لواحدة منهما فقط يضاف ازيد الصوديوم كمادة حافظة طويلة المدى من اجل الابحاث المستقبلية.الدراسة تضمنت ولاية الجزيرة وثلاثمحليات في ولاية الخرطوم هي شرق النيل ,الخرطوم وام درمان .التقييم المعملي شمل,اختبار نوعي مسحي باستعمال النشاط التثبيطي والطرق الميكروبيولوجية التي تم فيها فحص كل العينات لوجود بقايا المضادات الحيوية.سلالة من البكتريا العصويةالرقيقة تم استخدامها وتزريعها في وسط آجار غذائي. وضعت عينات الحليب في تجاويف في الاجآر .ورقة ترشيح واتمان المبتلة بعينة الحليب في طريقة اخرى.

تم استخدام مربع Chi- للمقارنة بين المحليات المختلفة , بين المزارع والاسواق وبين البقر والضأن عند مستوى احتمالية 5٪, لتحديد نسبة من بقايا المضادات الحيوية.من الخمسين عينة حليب 3(6٪)كانت موجبة لبواقي المضادات الحيوية .اثنتان من العينات الموجبة تم شراءها من الاسواق في محلية ام درمان بينما العينة الثالثة تم الحصول عليها من مزرعة في نفس المحلية. هذه الدراسة توصي بمسوحات اخرى للبقايا في مراكز تجميع الحليب وتقصي ممارسات انتاج الحليب بين مزارعي الحليب صغيري النطاق .هذا سيوفر اساس جيد لتصميم ممارسات مناسبة وفعالة لانتاج الحليب في المزارع صغيرة النطاق, للتقليل من تلوث الحليب والمساعدة على حماية صحة المستهلك في السودان.

# Chapter one

## Introduction

Realizing the concept of quality and safety of the milk sold to the market has ultimate benefits to the consumers and dairy products. Equal advantage obtained by producers of milk (farmers) and dairy industry by assuring safe milk is being processed to other products. For which this investigation is thought to visualize information to the chemical safety and quality of milk produced in Khartoum state and provides baseline data for further studies on milk safety.

The acquired information would also assist milk producers, Regulatory Authorities and consumers to contribute to the establishing of control strategies on the use of veterinary drugs in treating and preventing animal diseases. Use of Antibiotic that could result in deposition of residues in meat, milk and eggs must be forbidden in food intended for human consumption. If use of antibiotics is necessary, a withholding period has to be observed until the residues are few or no longer present. The use of antibiotics to bring about improved performance,synchronization or control of reproduction also results in harmful residual effects( Nisha ,2008).

A livestock census in Khartoum state, stated about (897,687 units animals), cattle are estimated at about (295,175) head, Goats (794,107) head, sheep (609,742) head and camel (295,175) head. (Databases of the Ministry of Agriculture, Livestock and Irrigation - Sudan) (2015).Khartoum State is considered as one of most significant Centers for milk and dairy products production, where methods of production differ from traditional production system (TPS) and modern dairy farm production system (MDFPS).

TPS is considered as the most popular system now a days for milk production and it includes:

(I) Back yard dairy unit

(II) Milk production unit around town& village (Dakkas)

(III) Small dairy units

M.D.F.P.S is very low in number. It presents pasteurized milk and other dairy products. It consists of the following companies &dairy farms:

(I) Arab company for milk& dairy products

(II) Blue Nile company

(IV) Kafouri dairy farm

(V) KuKu dairy farm

(VI) Khartoum State company for milk production

The gap in milk production iscompleted through exportation ofmanufactured milk powder and dairy products (AbdAlla, 2004). Sudan owns immense animal wealth which satisfies about 80% of local total milk need, (AOAD, 1992). Estimated milk yields in Khartoum State as 235 thousand metricTons .

**1.1.Research objectives:**

**1.1.1.General objectives:**

To determine the antibiotic residue levels in the raw milk produced by small-scale dairy farmers and the other sold in markets at Al-jazeera and Khartoum state, Sudan.

**1.2.Specificobjectives**

i) Qualitative screening of antibiotic residues in the raw milk of cows and sheep produced by farmers or sold in the markets of the study area.

ii) To evaluate awareness, attitudes and practices of farmers with respect to their usage and factors contributing to the presence of antibiotic residues in rawmilk.

## Chapter two

# Literature review

## 2.1.Milk

Milk is defined as the product from complete and uninterrupted hygienic milking of healthy, well fed and rested cows (Brasil.Ministerio da Agricultura,2013). Milk and milk products are a rich and suitable source of nutrients for people in many countries and there is a significant international trade of milk-based commodities. It is an important constituent of a balanced diet and is considered one of the world's most perfect foods and a rich source of proteins, vitamins and minerals, such as calcium, magnesium, phosphorus, potassium and zinc (WHO,2007).In the year 1999 the milk consumption in the state was estimated to be about 400000 tons, despite the fact that the actual production 360000 tons is produced in the state, of which almost 95% is produced from cows (Awad ,2006).

Temperate breeds are Kenana & Butana which are good milk yielding animals (Bayoumi, 1954). Also Boyns (1947) argued the potentialities of the Sudanese cattle as milk producers and reached to the conclusion that the Sudan possesses an excellent basis of cattle capable of rapid response to selection. Chemical composition of milk from temperate breeds holds an average milk composition as follow: Water 87.3%, fat 3.7%, protein 3-5%, total Solid (T.S) 12.8%, lactose 4.8%, solid non fat (SNF)9.1% and Ash 0.65% (Clarence *et al*,1982) whileThe average composition of cow's milk would be as follows: water 87%, fat 3.5-3.7%, lactose 4.9%, protien3-5% and ash 0.7% (Kon, 1972).

## 2.2.Biosynthesis of milk

The alveolus is the smallest complete unit of milk production and is spherical in shape with a central storage lumen surrounded by a single layer of secretory epithelial cells .Separation of basal membrane end from blood and lymph is by a basement membrane (Varnam and Sutherland, 1994) through which metabolites from the blood enter the secretory cell and are utilized in milk synthesis by the rough endoplasmic reticulum ,which emptys into the Gologi apparatus that transport the aqueous phase milk components to the lumen (Varnam and Sutherland, 1994).The lipids phase is also produced in the endoplasmic reticulum and collect on the cytoplasmic side of the membrane (Varnam and Sutherland, 1994).The lipid droplet pass into the lumen by the pinocytosis(Varnam and Sutherland, 1994).

Synthesis is finished in the alveolar lumen where lactose is synthesized and proteins glycosylated and phosphorylated while casein moleculesappear both in the Golgi vesicles and in the lumen (Varnam and Sutherland, 1994). The secretory epithelial cells are surrounded by a layer of myoepithelial cells thus when the circulating pituitary hormone, oxytocin is bound to these cells, the alveolus contracts and discharging the milk from the lumen into the duct system (Varnam and Sutherland, 1994).

**2.3.Adulteration**

According to US Department of Health, Education and Welfare (1953)it's defined as " any milk to which water has beenadded or any milk which contains any unwholesome substance, or does not conform with its definition(Siegnthaler and Schulthess, 1977). Developing countries suffers a lack of testing facilities and proper food legislation a situation expected to increase (Siegnthaler and Schulthess, 1977).

**2.3.1.Methods of adulteration**

Extraction of butter fat, addition of water , preservatives and coulouring matter (Shiegnthaler and Schulthess, 1977).Food and Drug Administration has ruled that milk containing antibiotics is an adulteratedbecause of the harmful effect to highly sensitive individuals(Sarrtwell, 1977).

**2.4.Antibiotics:**

Antibiotics are defined as naturally produced or laboratory synthetizedantimicrobial substances with the ability to inhibit or kill the microorganisms (Wageh *et al.*, 2013).They are used for many purposes resulting in formation of antibiotic residues in milk, if withdrawal periods are not adhered to .The frequent useof antibiotics may lead to veterinary drugs residues in the products of animal origin such as milk or meat(Kurwijila *et al.*, 2006; Mmbando, 2004).

Previous surveys pointed to the fact that these residues exist at levels below 1pgm (micrograms per gram), but even at these low concentration they might still have an influence on human gut microflora (Zwald *et al.*, 2004). The presence of antimicrobial residues in the foodstuffs of animal origin is one of the most important standard for their safety. Currently, approximately 80% of the all food-producing animals receive antibiotics for part or most of their lives(Pavlov *et al.*,2008).

**2.5.Classification of antibiotics and chemotherapeutic agents**

**2.5.1.functional classification**

Alexander (1985) reported that antibacterial agents are classified into three groups based on their activities:

**2.5.1.1.Broad spectrum antibiotics**: these are active against grampositive and gram negative (Ampenicillin and Tetracycline).Brander and Puch(1982) stated possible activity against rickettsiae, the larger

viruses ,and even protozoa and helminthes(e.g: chloramphenicol ,chlortetracycline hydrochloride ,oxytetracycline HCL and ampicillin).**2.5.1.2.Narrow spectrum:** mainly effective towards gram positivesuch as Penicillin (Brander and Puch, 1982) and Macrolides.

**2.5.1.3.Drugs works against aerobic gram –negative bacteria.2.5.2.Antibacterial Action**

**2.5.2.1.Bacterio static antibiotics**

Brander and Puch (1977) mentioned that all antibiotics are bacteriostatic in appropriate concentration resulting in stasis of bacterial growth in vitro; meaning that Invivo, the bacteria are made labile to body defense mechanisms such as: Sulphonamides, Tetracycline, Chloramphenicol and Erythromycin.

**2.5.2.2.Bactericidal antibiotics**

These produce actual death of the cell in vitro thus when used clinically they should produce their therapeutic effect without the help of body's defence mechanisms.These antibi*otics* involves:Penicillin, Streptomycin,Neomycin, Bactercin and Cephalosporins.

**2.5.3.Chemical classification**

Reilly (1977)classifications depending on chemical structure and purpose of action are:

2.5.3.1. Beta Lactams and other cell wall synthesis inhibitor (Gale, 1981) are Penicillin and cephalosporin,Bacitrein and vacomycin

2.5.3.2. Membrane active influencing permeability and causing leakage of intracellular constituents e.g polymyxins

2.5.3.3. Agents suppressing microbial protein synthesis

**(i)**macrolides:these agents owe large ring structureand result in reversible inhibition of proteins synthesis(chloramphenicol ,tetracycline)

**(ii)** aminoglycosides **:**which are composed of amino-sugar linked by glycosidic attached to various bases. The agent join the 30s ribosomal sub- unit thus leading to accumulation of protein synthetic intiation complexes.

2.5.3.4. DNA polymerase inhitor (Rifampin) working in nucleic acid metabolismand DNA Gyrase inhibitor e.g Quinolones.

2.5.3.5. Folate antiagonists(sulphonamide,trimethoprim):Antimetabolites which prevents specific step that are essential to micro-organsims.

**2.6.Absorption of antibiotic**

Inactivation of Teteracyclines is by iron,milk products and antacids (Davidson and Plumb,2003).Erythromycins (a macrolide) are unstable in gastric acidity when taken orally thus oral form must be used as acid resistant through itsadministration as a stearatesalt (Brander and Puch, 1982).Gentamycin is rapidly absorbed and readily distributed into various body tissues in less than an hour followingIM administration (Robbers and Tyler ,1996).Sulphonamides have a systemic sulfonamide (e.g. Sulphadimidine and Sulfadiazine ) which are well absorbed from intestine, and "Gut active" type (e.g. Sulphaquanidie) which are poorly absorbed from intestine ; (Brander and Puch, 1982).

**2.7.Interaction of antibiotics:**

**2.7.1.Synergistic**

discribes when the combined effect of two ormore drugs exceeds the algebraic sum of the effects produced by the drugsacting separately (Bogan and Yoxall , 1983) for example β -Lactam allows better penetration of aminoglycoside resulting in an overt Synergism(Robbers and Tyler, 1996).

**2.7.2.Antagonism**

Defines conditions in which the total effort of a combination of drugs is less than the algebraic sum of the effects of the individual drug in the combination (Bogan and Yoxall,1983).

**2.8.Metabolism and excretion of antibiotics**

Drugs are removed out of the body in an unchanged form or it is converted to another substances. These changes took place in the liver , kidney, or intestinal epithelium. The kidney secretes the unchanged drugs or its metabolites. A fixed proportion of drug is removed in a unit of time and it is called exponential clearance. (Archimbault 1983).Parke (1968) stated that on the whole system, these enzymes do not participate in the body's metabolism and are relatively un-specific. A good antibiotic shouldemerges out of the body in an unchanged form.Filteration of the drug in the renal tubules is through water reabsorption. (Bird &Nayler, 1971)**2.9.Veterinary antimicrobials**

It's defined as the primary group of veterinary medicinal products used since the 1950s as therapeutics for bacterial infectious diseases in both food-producing and companion animals. The substances applied belong to the same families as those used in human medicine (Sanders *et al.*,2011). These medicinal products are given to prevent and treat infectious diseases that could cause significant morbidity and possible mortality. The most commonly treated problems are digestive and respiratory system (Cazeau *et al.*,2010). For multiple types of integrated farm systems where animals (poultry, pigs, calves and fish) are raised in groups indoors, production conditions prompt veterinarians to prescribe these treatments for both preventive and curative actions. For other production systems, treatments are individual and mainly curative.

**2.10.Uses of antibiotics in animals production**

In veterinary practice antibiotics are used mainly for four purposes.There are three types of veterinary treatment (Sanders *et al.*,2011): preventive treatment (prophylaxis), given at a time in the animal's life when the risk of bacterial infection is considered to be very high; curative (therapeutic) treatment applied to sick animals; and control treatment (metaphylaxis) prescribed for groups of animals in contact with sick animals (Labro ,2012)and are thus expected that most of the group will become affected. With such treatment regimes, the antibiotics are commonly added to either feed or water. Antibiotics are also used in animals production as growth promoters to upgrade feed utilization and improve production(Katakweba *et al.,* 2012; Kurwijila *et al.,* 2006; Grane, 2000).

El Khawli (1999)reported that antibiotics might be applied bysome producer as milk preservative substances. Chemicals which has been used in as milk preservative include( boric acid, formaldahide, hydrogeinperoxide, hydrochloride and antibiotics (Hardiny and Ditton, 1995).Addition of antibiotics in milk for improving keeping qualities has been suggested (Saratwell, 1977).

**2.11.The commonly used antimicrobial agents in dairy Cattle :**

Antimicrobial agents are found in variable groups which are available for treatment of infected livestock. The most popular groups includes tetracyclines, beta-lactams, sulphonamides, aminoglycosides, macrolides, and chloramphenicol (Omore *et al.*, 2002; Movassagh and Karami, 2010; Pecou and Diserens, 2011). These antibiotics may be used alone or at times in combination when curing dairy cattle. These antimicrobial classes are extensively used as Medications in the livestock industry in Sudan in the most common cases such as mastitis.

In Tanzania studies conducted by (Kurwijila*et al.*,(2006); Mmbando, (2004); Nonga *et al.*, (2009); Midenge, (2011)) revealed that, tetracycline

particularly oxytetracycline, β-Lactam (penicillin G) and sulphonamide are the commonly administered veterinary drugs. It has also been reported that tetracyclines are the most popularly prescribed antibiotics in Africa symbolizing 41% of cases of antibiotic associated residues, followed by β-lactams at 18% ( Darwish *et al* .,2013).

**2.11.1.Tetracyclines**

Tetracyclines are a group of significant broad-spectrum antibiotics used in veterinary medicine to treat food-producing animals (Botsoglou and Fletouris 2001; Wang *et al.*, 2012). They treat gastrointestinal, respiratory, genitourinary and skin bacterial infections as well as infectious diseases of the musculoskeletal system and systemic infections, and also in the treatment of cholera and sepsis (Samanidou *et al.*,2007). However, they possess a range of side effects, including disturbances in healthy intestinal microflora, allergic reactions, liver and kidney malfunctions, hypersensitiveness and intense-light related dermatitis. Moreover, nowadays it is necessary to take into account the relatively high probability of acquired tetracyclineresistance (Michalova *et al.*, 2004).

The tetracyclines are bacteriostatic and are active against *Mycoplasma*, *Chlamydophila* and *Rickettsia* in addition to bacteria. Resistance to Tetracycline is now widespread among bacteria (Fuoco, 2012). Tetracyclines could be used parenterally, orally through feed or water or by intra-mammary infusion. The most popularly used oxytetracycline and the less often used tetracycline and chlortetracycline have similar properties. Fraction of tetracyclines execrated in bile gets reabsorbed through entero-hepatic circulation may persist in the body for a long time post administration (Chambers, 2006).

The rate of metabolism of tetracyclines in cows is approximately to 25-75 % and a significant percentage of the administrated tetracyclines are

excreted in bovine milk (Abbasi *et al.*, 2011). Photo-onycholysis and pigmentation of the nails may occur (Chambers, 2006). Photo-onycholysis is a phototoxic reaction leading to separation of the nail from the nail bed when treated individual is exposed to ultraviolet radiation. Tetracyclines can cross the placental barrier into the fetal circulation and amniotic fluid.

**2.11.2.Beta-lactam antibiotics**

It's a Group collectionincluding the ( penicillins, cephalosporins, carbapenems ,monobactam and others) making up the largest share of antibiotics used in most countries (Kummerer, 2009). They presents a broad spectrum type of antibiotics that act through interfering with cell wall synthesis and are used generally to treat Gram positive and Gram negative bacterial infections (Sun *et al.*, 2013). Among this group the penicillins and cephalosporins forms the major category used in veterinary medicine and are popularly used for the treatment of animals all over the globe.

Penicillins are the most frequently applied antibiotics for the treatment of bovine mastitis (Haapapuro *et al.*, 1997) .They do not get inactivated by pasteurization temperature or on drying and may thus cause allergic reaction appearing as skin rashes in very sensitive individuals even at very low concentration of 0.03 IU/ml (Bjorland *et al.*, 1998) to 0.01 IU/ml (Waltner-Toews and McEwen, 1994) in milk.

Cross reactivityis noticedbetween penicillins and cephalosporins for occurrence of allergic reactions. Meaning that Approximately 4 % of patients with a history of penicillin allergy suffers an anaphylaxis events to a cephalosporin too (Kelkar and Li, 2001) and patients with a history of a penicillin related allergic reaction have an exceeded risk of a reaction when given either sulfonamide or a cephalosporin (Apter *et al.*, 2006).Beta lactam antibiotics are sometimes accompanied by

neurotoxicity (Snavely and Hodges, 1984). Pre-existing brain lesions, renal dysfunction and hyponatremia might provoke these neurotoxic symptoms even at weak concentration of these antibiotics (Granowitz and Brown, 2008).

**2.11.3.Sulfonamides**

Derivatives of sulfanilamide are structural analogs of Para amino Benzoic Acid (PABA) and competitively suppress on enzymatic step (Dihydropterate synthetase) during which PABA corporates into the synthesis of diyhdrofolic acid (Folic acid). This result in inhibition of protein synthesis ,impairment of metabolic processes and suppression of growth and multiplication. They are best effective in early stages of acute infections when organisms are multiplying (Aiello and May, 1998).Sulfonamides are absorbed from the gastrointestinal tract (Burtis and Ashwood, 1991).Once there,it bounds to protein mainly toAlbumin of which About 60-90 percent is distributed to alltissues.

The metabolism of sulphonamide appears via N- acetylation. The product of metabolism owes no antimicrobial effect and it's finally dishcharged out of the body through urine, Bile and Feaces .Trimethoprim is antibiotic which was used to boost the effect of sulfonamide. It prohibits the reduction of dihydrofolic acid to tetrahydrofolic acid (Brooks,1995).

**2.11.4.Chloramphenicol**

Is a comparatively simple natural nitrobenzene derivative with a bitter taste. It is highly efficient and well tolerated broad-spectrum. Chloramphenicol suppresses protein synthesis by binding to 50s sub unit of 70s ribosome and impairing peptidyl transferase activity .it is originally bacteriostatic ,Although at high concentration may be bactericidal for some species (Aiello and Mays, 1998).

**2.11.5.Quinolones**

These are synthetic antibiotics (Reynold, 1989) .adminstered through 1/V, 1/M, and S/C they penetrate all tissues well and quickly. Some quinolones are eliminated un-changed e.g (ofloxacin), while others are partially metabolized e.g giprofloxacin and enrofloxacin and some are completely degraded. Metabolites are occasionally active.Main excretion through Renal and Biliary route (Cipprofloxacin and Nalidix acid). Quinolones appear in milk of lactating animals often at high concentrations that remains for some time.

**2.11.6.Macrolides Antibiotic**

They own a typical lactone ring in their structure (Tylosine&Erythromycin).They became concentrated in the biles and milk in which the macrolides concentration is several times greater than that of the plasma especially in case of mastitis infected cow(Aiello and May, 1998).

**2.11.7.Aminoglycosides**

known as a group of compounds, aminoglycosidesis are bactericidal and possess abroad spectrum activity against Gram +ve & Gram -ve bacteria (Singelton, 1995). It is comprised from Streptomycin, Neomycin-Framycetin, Gentamicin, Kanamycin and Tobramycin. Pyatkin and Kuvoshein (1980),declared that Streptomycin was obtained from *streptomycesgriseus.*Neomycin from *streptococcus Frachiae* (FAO, 1995).

**2.11.8.The polypeptide antibiotics**

Polymyxin are polypeptide antibiotics generated by different strain of Bacillus polymxa and it includes the following antibiotics : Bacitracin, Neomycin and polymyxin (Alexander, 1985).

**2.12.Residues:**

Residues of veterinary medicines are defined as pharmacologically active substances (whether active principles, recipients or degradation products)

and their metabolites, which persists in foodstuffs obtained from animals to which it has been administered (Codex Alimentarius , 2006).

### 2.12.1Marker residues

Marker residues are substances used to monitor the depletion of total residues in a food-animal tissues and to determine the target tissue FAO/WHO (2004).

### 2.12.2.History of Antibiotic Residues in milk

In some countries antibiotic usage in pin milk for improving keeping qualities has been suggested (Start well, 1977). In Zimbabwe 73 samples of raw milk from 3 main dairy market board collection centres, were assessed for the presence of microbial growth inhibitory substances. 4.4% of these samples were found postive (Chagonda and Ndiku wera, 1989).

In Malaysia Salam *et al* (1991) tested 66 fresh milk samples from three small holder dairy farms for the presence of antibiotic residues.

In Lisbon 2248 samples of consumer milk were analysed in 1981 to 1985. Six hundred and seventy four of them 30% contained residues(Barbosa *et al* 1991). In Estonia, Paern and kind (1995) assessed 47 raw milk samples sold in Tartu for the presence of antibiotic residues, the residues were detected in 4(8-5%) samples.

A qualitative receptor assay for antibiotic and antimicrobial residues in milk was utilized in a survey of commercial milk samples collected from eastern Pennsylvania, Central New Jersey, New York City area. Sixty-three percent of milk samples contained one or more residues; 27% contained 2 residues; 11% contained 3 or more residues. Tetracyclines and sulfonamides were the most detected (Brady and Katz,1988).A study aiming to detect betalactam and sulfonamide residues in milk through "ELISA" screening method and "HPLC" confirmatory method proved that of 127 samples of milk analysed, over 70% of them (64 were

contaminated with betalactam residues and 24 with sulphonamide residues) contains residues of drugs.(Sulejmani *et al*.,2012).

The most recent study till now were that carried out by Husnain *et al* (2017) to determine the present of β- lactam (Penicillin G, Ampicillin, and Amoxicillin) antibiotic residues in 120 samples of unprocessed market milk through Qualitative assessment using *Bacillussubtilis* Field Disc Assay followed by Quantification assessment of positive samples using (HPLC). Resulting in 28 positive samples that showed (ampicillin, amoxicillin and penicillin )existed in 32 % (9/28), 85% (24/28) and 89% (25/28) of positive samples, respectively. Orwa *et al* (2017)conducted an investigation in Nakuru County for the occurrence of 13 veterinary drugs of tetracyclines and sulphonamides using Charm II Blue -Yellow-test and HPLC-UV as a confirmatory test .In the rural areas (31.4%) samples were positive whereas only (23/80) of the samples reacted positively In the peri urban areas.

In Sudan Barakat (1995) applied delvo test P for the detection of antibiotics residues in 80 milk samples, he found that 8.75% gave positive results. Mustafa(2002) examined 100 milk samples & he got negative results in all of them. Osman (2002) reported that the percentage of positive samples for total samples examined was 0.8% and for the samples taken directly from the udder, it was 4.0%.In Sudan, delvo test SP was carried out to detect antibiotics residues in 236 milk samples and it was found that about 21.18% gave positive results (Omer, 2016). In Khartoum state 64 milk samples were assessed for the presence of neomycin and tylosin and the result came positive for all f the collected samples 100% (Elhassan, 2012).In Khartoum state,Sudan a total of 734 raw milk samples collected in order to detect antibiotic residues. Penicillin was found to be used in 61.7% of the farms,while tetracycline in only 27.7% of the farms.( Salman*et al.*,2012).

**2.12.3.Concern over antibiotic residues in food of animal origin:**

Concern over residues in food occurs in two occasions; one is producing potential toxicity in human, and the low levels of antibiotic exposure would result in development of resistant strains which cause failure of antibiotic therapy later on( Nisha ,2008).

The initial concerns was expressed by the dairy processors who reported that contaminated milk suppressed the starter cultures used in the production of fermented milk products and affected the results of the dye reduction tests used for milk quality at the time(Mitchell *et al* .,1998).**2.12.4.Source of drug residues:**

Drugs are applied to dairy cows for treatment of mastitis through intramammary or intravenous infusions and for disease therapy by intramuscular or intravenous injections, oral administration, feed supplementation, or reproductive infusions. FDA surveys points that improper use of drugs in the control of mastitis is the major source of residues found in the milk supply.

The beef industry has allegation that a great percentage of the drug residues found in beef-carcasses are in those of culled dairy cows. Many drugs are preserved in the animal body for longer times than indicated by label discard times. Consequently, milk samples remain positive for residues. A good example is penicillin whose recommended milk discard time of 72 hours. However, penicillin residue has persisted in milk for as long as 18 days(Jones,1999 ).

**2.12.5.Suspected reasons for drug residues include:**

Extended usage or excessive dosage of conformed drugs ,weakness in recording treatment , accidental pouring into bulk tank ,failure to follow recommended label withdrawal time ,lack of awareness on withdrawal period ,prolonged drug withdrawal ,problem in identification of treated-

animal ,multiple dosing ,not following the label direction in using the drug, prohibiting milking from treated quarters only, filthy milking equipment, early calving or narrow dry periods ,buying treated cows and use of dry cow treatment to lactating cows (Jones,1999).

Drugs administered for dry cow treatment do not appear to cause drug residues if milk is not shipped for the first four days after calving, if dry periods are longer than six weeks, and if dry cows do not get into the milking herd. If manufacturer's recommendations are obeyed , dry cow therapy should not result in residues aftercalving. However, residues are possible and fresh cows must not be tested, especially cows with short dry periods ( Jones,1999).

Formulation and route of administration can have strenuous effects on the pharmacokinetics and tissue residues of a drug. Proprietary differences in formulations, even in the same drug, leads to illegal residues if not used according to label instructions. Extralabel use of medications in food animals is forbidden except if there is no approved medication or if the approved one is useless ( KuKanich,2005). Milk Samples collected at 24 h intervals through 120 h after treatment from lactating dairy cows. Antibiotic residues were determined qualitatively by microbiological assays using *Bacillus stearothermophilus.* Intrauterine infusion of antibiotics resulted in the lowest percentage of milk samples positive for residues while the high percentage of samples was positive for residues were after intramuscular injection of antibiotics; Nevertheless, most samples were negative by 72 h after treatment. Intramammary therapy had the high proportion of samples positive for residues at 24 and 48 h after treatment, and some samples were even positive 96 to 120 h after treatment. Samples from treated quarters were usually positive when corresponding composite milk samples were negative. Treatment with

multiple antibiotic through different routes resulted in the highest percentage of samples positive for residues for the longest time (Oliver *et al.*,1990).Recommended use of on-farm drug residue testing including drug withdrawal period, milk discard time, testing of treated cow post milk withdrawal time,comfirmatory testing for positives cows,not treating cow with a poor chance to respond ,testing of culled cows ,calves suckling on a treated mother ,newly purchased cow and first lactating cow's milk before adding it to the bulk milk tank (Jones,1999).

There are various problems in this field such as the increased number of new substances in the 'black market' every year to be used as growth promoters as observed in the high competitive sports. Another problem is mixing of low amounts of multiple substances, like a 'cocktail' that exerts a synergistic effect.Finally, the development of interfering substances to mask immunoassay detection systems hindering the efficient detection of the illegal substances. In addition, control laboratories face more strict needs for the performance of analytical methods according to new directives because of the large number of samples to analyse, large variety in samples and residues to be examined, requirement for adapting analytical methodologies to new Directives with strict guidelines, the increased costs in developing such new methodologies, the multiple residues to search per sample and the need to invest on strong new instruments. The availability of screening methodologies decreases the number of samples to be confirmed through costly and difficult confirmatory analysis. Recent developments will probably be routinely implemented in the upcoming few years( Toldra´ and Reig,2006).

### 2.12.6.Factors affecting drug residues

1. Hapke and Grahwit (1987) confirmed that the concentration of

drug in animal tissues is directly correlated to the absorbed dose.

2. The route of drug administration, intramusular and subcutaneous injection results in high concentration and persistence of drug residue at the site of injection (Standers *etal*, 1988).

3. Sumano *et al* (1990)accomplished that the drug clearance in healthy and diseased animals are not the same. In diseased animals, residue can remain two or three times longer than in healthy animals.

4. Drug formulation affecting residues Baggot, (1992) reported that the only preparation of drugs are delayed in clearance is those afterlocal intramuscular injection.

5. Baggot, (1992) also stated that different antibiotic types differ in their residues.

6. Katz & Brady (1993) issued that deposition is the reason for varying concentration in different tissues, high concentration must be expected in excretory organs.

**2.12.7.Pathological Effects elected by Antibiotic Residues:**

Occurrence of antibacterial residues in animal originated foodstuffs exposes the consumers to health risk such as :

1. Antibiotic residues in food are potential threats due to their direct toxicity in human and their low levels would result in death of intestinal flora, cause disease and other problems such as development of resistant strains(Nisha 2008;Heshmati *et al.*, 2015).
2. Immuno-pathologicaleffects, Autoimmunity,Carcinogenicity due to (Sulphamethazine,Oxytetracycline&Furazolidone),Mutagenicity,Nephropathy(Gentamicin),Hepatotoxicity,Reproductive disorders,Bone marrow toxicity (Chloramphenicol),Allergy (Penicillin)( Nisha ,2008)and goitrogenicity (Kinsella,2009)risks have also been observed.

3. The use of antibiotic in humans will be rendered ineffective(Weaver, 1992).
4. Residues of antibiotic may inhibit acid production by starterbacteria and significantly affect cheese making processleading to longer making time and disruption of cheese makingschedules. Also inhibit strain of streptococcus thermophillusused in yogurt manufacture.
5. Aminoglycoside: cause acute tubular necrosis when used inhigh dose i.e. in a dose more than 35 Microgram per milliliter.

**2.13.Withholding time**

Withholding time is the peroid of time during which the product continues to be excreted in the milk after the last day of administration (WHO, 1970).

**2.14.Acceptable daily intake ADI**

To explain the level of risk of any pesticide, its actual exposure is compared to a reference safety threshold, e.g., ADI; calculated for experimental animals and extrapolated to humans. ADI is the quantity of a substance, expressed on a body-mass basis, daily ingested in food or drinking water over lifetime without imposing any appreciable risk to human health (WHO, 1987). The calculation to set the ADI is relays on one hundredth (1/100) the dose considered to be non-toxic in animal feeding trials; toxicologically known as NOAEL (Faustman and Omenn, 2001).The ADI is calculated using the observable effect level (NOEL) or the dosage level (mg/kg) at which no adverse effects are observed as established by animal bioassay toxicological studies.

**ADI** (mg/kg/ day) =**NOEL/SF**

**SF:** Safety Factor

Varies 100-1000 based on the use of the drug in question and the amount and degree of toxicity data presented by the manufacturer.

**2.15.Maximum residual Limit MRL:**

The Codex Alimentarius and Joint FAO/WHO programme have been formulating the standards concerning the residues in foods since 1985. For the international registration of veterinary drugs in the EU, the Committee for Medicinal Products for Veterinary Use (CVMP) has been developed. CVMP, depending on the toxicological residue assessment, sets the MRL levels for the pharmacologically active chemical agents of the veterinary medicinal products occurring in foodstuffs. The establishing of the MRL level in the EU is organized by the Council Regulation (EEC) 2377/90. All veterinary drugs at the European market distend for food animals must be toxicologically assessed and categorized into Annexes I–IV. Depending on the MRL type.

MRLs present the internationally acknowledged limits which determines maximum quantity of the drug residues that may be found in foodstuffs of animal origin. According to the Commission Regulation No. 1662/2006, food business operators should introduce procedures ensuring that raw milk will not be marketed if it contains the residues of antibiotics in amounts overcoming the levels for any of the substances authorised in the Annexes I and III of the Regulation (ECC) No. 2377/90, or if the overall content of all antibiotic residues overcomes the maximum residue limits (Navratilova,2008).

Aiming to prevent any harmful health effects on consumers, Food and Agricultural Organization, World Health Organization and European Union (EU) have established the maximum residual limits (MRL) for veterinary drugs (Council Regulation 2377/90/EEC). The maximum residual limit set by the EU legislation for tetracycline (TTC), oxytetracycline (OTC) as well as chlortetracycline (CTC) in raw cow

milk is set to 0.1 mg/kg (100 ng/g) (Navrátilová *et al* .,2009)as illustrated in table(1.1).

Legislation establishes the MRL for three tetracycline antibiotics most commonly utilized in lactating dairy cows. The MRL for tetracycline (TTC), oxytetracycline (OTC) and chlortetracycline (CTC) in cow's milk is 100 μg·kg-1 (Commission Regulation 37/2010). When heated or exposed to acidic or highly alkaline environments, tetracyclines are exposed to chemical transformation processes, such as isomerization and epimerization (Wang et al. 2012).And that is why when establishing MRLs it is necessary to take into account both the basic compound (tetracycline) and its epimers (the 4-epimer products of TTC, OTC and CTC) (Commission Regulation 37/2010; Spisso *et al.*,2010).

**Table(2.1).MRL for Some Veterinary Drugs in Milk.**

| **Antimicrobials** | **MRL (μg/l)** |
|---|---|
| Teteracyline | **100** |
| Cholorocycline | **100** |
| Oxyteteracyline | **100** |
| Doxyteteracycline | **100** |
| Benzyl pencillin(procaine) | **4** |
| Ampicillin | **4** |
| Amoxicillin | **4** |

| | |
|---|---|
| Cloxacillin | **30** |
| Dicloxacillin | **30** |
| Oxacillin | **30** |
| Streptomycin | **200** |
| Erthromycin | **4** |
| Gentamycin | **200** |
| Tylosin | **100** |
| Lincomycin | **150** |
| Monensin | **2** |
| Sarafloxasin | **100** |
| Spectinomycin | **200** |
| Sulfamethazine 25 | **25** |
| Sulfadimethoxine 25 | **25** |
| Sulfamerazine 25 | **25** |
| Sulfathiazole 25 | **25** |
| Sulfamethoxazole 100 | **100** |
| Sulfanilamide 100 | **100** |
| Sulfadiazine | **100** |

**Source:**FAO/WHO-Codex Alimentarius Commission: Maximum Residues Limits (MRL) for Veterinary Drugs in Foods- CAC/MRL 2-2012 Standard.

### 2.15.1.Examples of some countries with residual values exceeding the MRLs:

According to the European union and Codex Alimentarious regulation for maximum residual limits, sulfonamides should not exceed 100 μg/kg and tetracyclines should not exceed 100 μg/kg (EUR-Lex 2010). Antibiotics have been reported in values above the standard residual limits in countries such as: Germany (Kress *et al.*, 2007), Netherlands (Abjean *et al.*, 2000), Mexico (Tolentino *et al.*, 2005) Turkey (Alkan, 2007) among

others. In Africa, countries identified to have milk contaminated with Antibiotic residues includes: Egypt, Ghana, Ethiopia, south Africa, Nigeria, Tanzania and Sudan (Myllyniemi *et al.*, 2000; Kurwijila *et al.*, 2006; Goudah *et al.*, 2007; Addo *et al.*, 2011; El-tayeb *et al.*,2012).

In Kenya in the autumn of 2010, 2.5% and .6% samples contained sulphonamides and tetracyclines respectively (Ahlberg *et al.*, 2016). High levels of tetracyclines were obtained in Algerian milk and milk products in study by Layada*et al.*,(2016). Chowdhury *et al.*, (2015) reported levels of antibiotic residues above recommended limits in Bangladesh milk. Over 60% of milk samples contained antibiotic in Nigerian milk and other milk products (Olatoye *et al.*, 2016).

**2.16.Techniques for Detection and Analysis of Drug Residuesare**:

Variable methods and assays for the detection of residues of antimicrobials, mainly in cow milk, have been developed and validated, whereas few studies have been performed so far for the finding of residues in sheep and goat milk (Wang *et al.*, 2006; Comunian *et al.*, 2010).These detection methods are either screening methods or chromatographic methods the later detects multiple antibiotics even at low concentrations. The screening tests are mostly carried out through microbiological (Nouws *et al.*, 1999; Babapour *et al.*, 2012), enzymatic and immunological methods (Strasser *et al.*, 2003).

The basis of screening methods depends on the different susceptibility of bacteria to variable antibiotics. The antibiotic residue detection assays that are now available utilizes variable methods and test microorganisms (Mitchell *et al.*, 1998). Microbiological assays for the finding of antibiotic residues use bacteria such as *Bacillus stearothermophilus* or *Bacillus subtinus* because of its high sensitivity to the most antibiotics. The first test for constituting antimicrobial residues in milk (microbial inhibitor test) was progressed as early as 1952 (Mitchell *et al.*, 1998). The

developments of tests for detection of antibiotic residues were initiated to determine the inhibitor agent levels in milk, since the presence of these agents might cause the inhibition of the starter cultures of dairy industry (Navratilova, 2008).

These methods are comparatively cheap, simple and capable of detecting an extended diversity of antimicrobials. An obstacle which limits their use is a long incubation period. For which, rapid assays have been developed which authorize acquiring the results rapidly.These rapid tests are simple to carry out, sensitive and specific. It includes Penzyme test which was established in 1980's. Later on, in 1988, Charm II test for detecting 7 types of antimicrobial agents was introduced to the market, accompanied later by other rapid assays, e.g. the LacTec test (1991), SNAP test (1994), Beta Star test, Charm Safe Level test (Mitchell *et al.*, 1998) and Charm MRL-3 (Reybroeck *et al.*, 2011; Fejzic *et. al.*, 2014).Also Elisa, Hplc, Liquid chromatography,Gas chromatography and Paper chromatography ( Nisha ,2008). Nevertheless,there are wide range of techniques applied for detection of residues in milk matrix that vary extensively based on the available facilities,techniques adopted and the most important sensitivity of the test.

**2.16.1.Biological Methods**

Include microbial inhibition and enzyme-linked immune sorbent assay (ELISA).

**2.16.1.1.Microbiological methods**

Silver man& Kosikow (1952)developed this method. bacterialgrowth inhibition methods were extenseively performed as screening methods for detecting antibiotic residues. A number ofmicrobiological assays for detecting antibiotic residues have been developed as in1941, the cylinder plate assay method was first described, between 1944 and1945 ; the filter paper disc method was introduced (Bishop *et al.,* 1992). However, they

mentioned that since 1950s the *Bacillus subtilis* disc assay method andit'smodifications have been used to detect residual antibiotics in milk and during the1970s, the disc assay and the tube assay methods that use the *Bacillusstearothermophilus* organism gained acceptance and broad usage.

The Microbiological tests are sheaf, easy to carry out on a large scale andthey possess a wide, non specific in sensitivity (Nouws *et al.,* 1999) .Several studies have shown that false-positive results occurred on samplescontaining no drug when using the delvotest assay; one of the microbialgrowth inhibition assays; which is a simple, sensitive and broadly drug-detectingtest system (Andrew, 2001).Microbial growthinhibition methods make the benefit of a standard culture of the tested microorganism inliquid/solid medium (Heeschen,1993).e.g.Geobacillus stearothermophilus var. calidolactis ,*Bacillus subtilis*, Bacillus megaterium, Sarcinalutea, Escherichia coli,Bacillus cereus var. mycoides or Streptococcus thermophilus.

The analysed milksample is applied on the agar surface either directly or with a paper disc (disc assayplate methods). In the course of incubation, the diffusion of the sample into themedium takes place (the agar diffusion principle) and if the sample containsinhibitor agents, prohibition or total inhibition of the tested microorganismgrowth occurs. Depending on the method used, the existence of inhibitor agents in thetested sample is indicated by the formation of aclear zone of inhibition around thedisc (disc assay plate methods) or a change in the medium colour (Botsoglou and Fletouris 2001).

Microbial growth inhibitionmethods (wides pectral rapid tests) differ in the type of the testing organism,indicator, incubation period and temperature, spectrum and detection levels of theagents analysed. A series of these methods utilize the testing microorganismGeobacillus

(Bacillus) stearothermophilus var. calidolactis: BR-test/AS/BlueStar/6/7 (Enterotox Lab., Germany), CharmBlue Yellow Test (Charm SciencesInc., USA), Delvo test SP-NT (Gist-brocades BV, The Netherlands),CMT – Copanmilk test (Copan Italia,Italy), Eclipse 50 (Zeu-Inmunotec S.L., Spain).Geobacillus stearothermophilus is a remarkable testing microorganism for its propertiesfromwhich the most important, according to Katzand Siewierski (1995), are: theability of rapidgrowth at higher temperatures (64°C) and a highsensitivity to the β-lactam antibiotics .

Commercially available microbial inhibitor assays play animportant role in the integrated detection system. At present, many commerciallyproduced microbial inhibitor tests are done simultaneously with selective rapidtests formilk screening in primary production, in dairy industry and in accreditedlaboratories (Suhren1995;Honkanen-Buzalski and Reybroeck 1997;Honkanen-Buzalski and Suhren 1999; Botsoglou and Fletouris 2001).The advantage of thesemethods is that they have an extended detection spectrum; simple to carry out,and not costly and can be used for the screening of a large number ofsamples (Mitchell *et al.*, 1998).These methods have their disadvantages, however, thatlimit their use: they do not enable specific antibiotic identification, havelimited detection levels for a series of antibiotics ,regarded as qualitative only andrequire a long incubation period (2.5–3.5 h). They are highly sensitive to β-lactamantibiotics, mostly penicillin, but approved less sensitive to other antimicrobialagents such asmacrolides, sulfonamides, tetracyclines, orchloramfenicol(Botsoglou and Fletouris 2001). Many studies confirmed that natural antimicrobialagents,if present in milk in higher concentrations, canbring about false – positiveresults (Andrew 2001; Kang & Kondo 2001;Kang*et al.*,2005).

Commercially generated microbial inhibitor tests are delivered in the form ofampoules (mono tests)or in the form of micro-plates with a high number of testingcells. Apart from water bath or incubator, they do not request a special laboratoryequipment. To avoid subjective variations in the visual interpretation and to takethe readings in an automated and more objective manner; some authors performingphotometric measurements use the appropriate Wavelength (590 nm) and anotherwavelength asreference (650 nm) in ELISA reader (Althaus *et al.*,2003). Whenperforming microbial inhibitor tests, it is a must to meet the standards of goodlaboratory practice (protection against the contamination of the test), checking thepH value of the sample, observing carefully the correct temperature and theincubation period as specified by the producer's instructions and testing a positiveas well as a negative control alongside with the sample. Some of the microbial inhibitorscreening methods, in frequent use are, for example: Eclipse test, Charm Cow sidetest, Charm AIM-96, Charm Farm test, VALIO T101, Copan Milk test, and others.

The four plates assay was atypical bacterial inhibition test. In this method discs of tissue are placed on four agar plates inoculatedwith microorganism and the plates are then incubated under varying conditions to allow inhibition of growth by a diversity of antimicrobial drugs (Dixon *et al*., 1993). Apositive result is decided by complete inhibition of growth on the surface of the medium in a zone not less than 2mm wide around the tissue disc.The inhibition assays necessitate the preparation of Muller Hinton Agar in sterile glass plates, thereafter uniform streaking of *B. subtilis*, followed by creating wells/holes on the media using sterile boring glass rods. After which 10µl of sample pipetted in the wells and the plates incubated at temperature of 37 $^{0}$C for 18-24hours. Following incubation the cultures examined for bacteria growth inhibition zone .In

case of antibiotic positive results;The dimensions of the inhibition zones are measured with callipers. Testing of milk and other animal food samples for the presence of antibiotic residues is usually performed with the help of microbial inhibition assays. Their sensitivity to different drugs relays on the indicator microorganism used and the concept of the test. Microbiological assays for the detection of antibiotic residues uses the genus *Bacillus,* due to its high sensitivity to the majority of antibiotics (Jevinova *et al.,* 2003).

**2.16.1.2.Enzyme linked immunosorbant assay (ELISA)**

ELISA is highly specific and easy to apply from simpleextraction procedures and rapid reaction time as the results from ELISA are available in less than one hour and large number of samples could be tested for antibiotic residues. However, wide ranges of ELISA tests were needed to test for all possible antibioticsand cross reaction with metabolites and compound withsimilar structure prevents accurate identification .So confirmation test with massspectroscopy or high performance liquid chromatography (HPLC) are requested(Patal and Bond, 1996)

**2.16.2.Chemicals methods**

These methods are comprised from high performance liquid chromatography (HPLC),mass spectroscopy and thin layer chromatography (TLC). They can differentiate between variable antibiotics (Patal and bond, 1996). HPLC is expensive, requires different techniques to deal with different antibiotics, other chemical methods like thin layer chromatography (TLC) were also practiced, it supplys a solution to conduct simple& cheaper techniques but they were limited by the complex extraction and clean up protocols. High voltage

electrophoresis bio-autography was utilized for identification of sulphamethazine and penicillin in milk. They extracted the antibiotics through employing acteonitrite while thin layer, electrophoresis uses an agar medium seeded with microorganism.

**2.16.3.Electrophoresis**

High voltage electrophoresis bio-autography wasexecuted for identification ofsulphamethazine and penicillin in milk (Loit and Vaughan, 1985). The antibiotics are extracted through acteointrite and then electrophoresis is performed using agarmedium seeded with the microorganism

**2.17.Residues control methods:**

In the EU, self-monitoring and the control of residues relays on standardized analytical methods. Much of this analysis is performed in the laboratory. The regulatory framework implemented in the EU is based on Directive 96/23/EC, which structures the network of laboratories approved for official residue control, laying down requirements in terms of quality and performance of analytical methods(European Commission,2002). This framework has participated in the harmonisation of controls. Conversely, in UEMOA countries, the list of references of harmonised analysis methods for food did not consist of any methods for analysing veterinary medicinal products.

Analysis methods differs from one country to the next, and even among laboratoriesl; due to the lack of UEMOA-accredited methods. Against a background of trade globalisation, analysis methods must be standardized and carried out by all laboratories, with equivalent levels of performance. In general, the residue control strategy depends on two-step approach: the detection of residues through sensitive tests with a low rate of false negatives; after which comes confirmation, requiring quantification

against the MRL and identification with a low rate of false positives(Mensah *et al*., 2014).

### 2.17.1.Control plans for antimicrobial residues in milk

In the EU, processors frequently performs controls for antimicrobial residues and there are systematic checks of bulk tankers to screen for the presence of inhibitors(.European Commission (EC) ,2010). The lack of inhibitors is a quality criterion that increases the price that a farmer receives for milk. This is without doubt the reason why rates of non-compliant residues in milk are very low in the EU. Very few studies have been directed to evaluate antimicrobial residues in raw milk in African countries, with the exception of those in North Africa, because milk is not a staple food in these countries (Donkor *et al* .,2011).

Tetracycline (TC) residues are classified as relatively unstable compounds. Temperature during cooking has the greatest impact on the loss of tetracycline residues (Abou-Raya et al., 2013; Hassani et al., 2008;; Loksuwan, 2002) and among these different studied cooking procedures, microwaving was the most effective .

If cooking temperature and time are enough, we ensure great losses of TC residues. Therefore cooking provides safety margin for products containing TCs (Hassani *et al.,* 2008).nevertheless, pharmaceutical drugs – antibiotics that are used in humans must not be used in animals too and their provision must be limited to a reasonable and allowed level(Sulejmani *et al.*,2012).

# Chapter three
# Material and Method

## 3.1.Study Area

The study was conducted between november 2017 and April 2018 in Aljazeera state and3 localities in Khartoum state, Sudan. Khartoum state the capital and the Largest city in Sudan .It is located at the confluence of

the white Nile ,flowing north from lake victoria ,and the Blue Nile ,flowing west from Ethiopia.

The location where the two Nile meet is known as "al-Mogran".It's located in the middle populated area of Sudan ,at almost the northeast center of the city between 15 and 16 degrees latitudes north and between 31 and 32 degree latitude east. Khartoum is relatively flat ,at elevation 385 m(1,263ft),as the Nile flows northeast past Omdurman to Shendi at elevation 364 m(1,194ft) about 101 miles (163Km) away. It is classified under the Koppen's climatic system, featuring a hot arid climate. Themean annual minimum and maximum temperatures are 15 $^{0}$C and 37,1$^{0}$C,respectively. The mean relative humidity is 21%.

**3.2.Study population**

The study population consisted of cows and farmed sheep .milksamples were boughtfrom Al-jazeera state and either bought or gathered from the 3 localities in Khartoum state.

**3.3.Materials**

**3.3.1.Test medium**

Mueller-Hinton agar in the form of dehydrated powder was used. The medium formula per liter contains:

0.2 g beef extract

17.5g casein hydrolysate

1.5 g starch

17.0 g agar

PH adjusted to neutral 25$^{o}$C(Mueller and Hinton ,1941).

This media is characterized with few properties making it excellent for antibiotic use :

1.it's a non selective non deferential media meaning that all organisms plated will grow equally

2.itcontains starch a substance well known for it's inclinations towards toxin absorptions thus bacterial toxin can't interfere with the antibiotic

3.it's a loose agar ,which allows for better diffusion of the antibiotic than most other plates leading to a truer zone of inhibition

4.It shows acceptable batch to batch reproducibility for susceptibility tests.

5.It's low in sulfonamides ,trimethoprim and tetracycline inhibitors such as Para amino-benzoic acid (PAPA) ,thymidine and thymine making it suitable for susceptibility tests to these antimicrobials(Mueller and Hinton ,1941).

**3.3.2.Solutions**

**3.3.2.1.Distilled water**

It was obtained from theVeterinary Research Institute (VRI)

**3.3.2.2.Normal saline**

It was prepared by dissolving 9g of sodium chloride in 1000 ml distilled water and sterilized at 121ºC to 15 Ib/sq inch for 15 minutes, and cooled.

**3.3.3.Test organism**

*Bacillus subtilis* used for this study was obtained from theVeterinary Research Institute (VRI).

**3.4.Methods:**

**3.4.1.Sample Size**

Fifty samples werecollected from Al-jazeera state and each of the 3localities covered in Khartoum state . Each sample was collected twice (once with and once without Sodium azide)giving a total of 100 sample containers.

**3.4.2.Sampling Procedure**

Fifty milk sample were collected from Al-jazeera and Khartoum state the amount of milk sample was randomly selected and collected from each farm individual sheep/cow and market bulk milk tank .In the same area each sample was divided into two separate sterile plastic containers 50 ml in diameter. Sodium azide was added to only 50 ml of each sample as a bacteriostatic agent. General labeling information were acquired by asking the dairy farm owners in case of farms or the seller in case of markets. These information including(Date, Animal species, Antibiotic injected or not, Locality , identification code/number and whether it contains Sodium azide or not ). All the samples were immediately chilled in ice-containing thermos and preserved once arrived into deep freezer at -20 $^{0}$C until processing or analysis.

**3.4.3.Localitions and sources of the samples:**

Three Localities in Khartoum state were chosen namely(East Nile, Al-kharotum and Omdurman) and Al-jazeerza state.

**Table(3.1).Locations and sources of the samples:**

| **Location** | **Farm sample** | **Market sample** |
|---|---|---|
| East nile locality | 26 | 0 |
| AL-khartoum locality | 0 | 3 |
| Omdurman locality | 4 | 7 |
| Al-jazeera state | 0 | 10 |

**3.4.4.Sample Processing**

**3.4.4.1.Preservation :**

The processing of the sample containing Sodium azide was done as soon as possible through De-fattening using a Centrifuge to reduce the bacterial growth and preserve the samples.The procedure consist of defrosting the samples over night on the previous day.each sample content was divided in three centrifugation tubes, labeled ,balanced with

other sample ,rotated at 5000 rph for 20 minutes then cooled in a Deepfreezer at -20 $^{0}$C for 20 minutes; to allow for the supernatant separated top fat layer to solidify for facilitating it's removal with a sterile plastic stick or removing the milk under it with an aid of a syringe .

**3.4.5.Media preparation:**

38 g of the medium was suspended in one liter of distilled water.It was heated, agitated and kept boiling for one minute to ensure that it was completely dissolves . Followed by autoclaving at 121$^{o}$C for 15 minutes for sterilization .Then it's was cooled and poured into disposable petri-dishes on ahorizontal surface level ;to give a uniform depth. The thinner the agar layer the better detection of positive samples. After that it was allowed to cool at room temperature, and checked for final PH 7.3 ±0.1 at 25$^{o}$C . Finally it was stored in the incubator over night to dry .

**3.4.6.Screening of samples:**

For each sample a qualitative test was carried out through the microbial inhibition test using *Bacillus subtilis* Field Disc Assay in two stages.This bacteria is highly sensitive for multiple antibiotic including Pencillin and Tetracycline.Media chosen was Muller Hinton media because it's a selective media for *Bacillus subtilis* thus inhibiting growth of other contaminats.Each stage consist of three day work .

**3.4.6.1.The first stage**

It is focused on pure milk samples(samples without sodium azide as an initial rapid screening).On the first day a fresh *Bacillus subtilis* subcultured plate was prepared by four way streaking of the raised,dull, wrinkled colonies of pure *B. subtillus*(as shown in figure (3.1)concurrently on the same day the preparing ,sterilizing and pouring of the media was done too.On the second day one pure colony of the

bacteria was inoculated into sterile normal saline ;to obtain optical density value of 0.5.The required density was achieved through grossly comparing the diluted colony with the density of Mac-Forland solutionsunder a strong light(as illustrated in figure (3.2)).

Each petri-dish was labeled,the falcon containing milk sample was shaken and a swap was dipped into the sample and a one direction streak was made;to avoid the very thick growth criteria known of this bacteria that prohibit or inhibits the detection of inhibition zone of antibiotic containing sample.Each disc was divided into two half to examine two sample in each .A two well were made in each disc of equal distances from each other and from the wall of the dish .A well was made for each sample with the help of a suitable punching machine(ie:2 wells in each petri-dish one for each of the two samples).A drop of milk from each sample were put in a separate well. Antimicrobial susceptibility standards test discs of required antibiotic were made concurrently for the control purpose. The Discs were then incubated at 37 $^{0}$C for 24 hr on acidic condition( PH of 0.6) to test for the antibiotic residue. The positive results were manifested by formation of transparent zones around the well (as shown in figure (3.3))and the zone of Inhibition for each well was examined and measured separately(as illustrated in figure 3.4))(Jevinova *et al.,* 2003).

**3.4.6.2.The second stage**

This stage is similar to the previous process except that each sample was cultured in a separate plate ,for both those without or with the sodium azide ;for validation .Because sodium azide is considered a preservative thus prohibits bacterial growth as shown in figure (3.5).It includes the well method and milk sample-impregnated Whatman's filter paper

method .The filter paper procedure consist of 6 mm diameter Whatman's filter paper made with the help of punch machine. The falcon tubes containing milk samples were shaken and the prepared discs were dipped multiple times into the samples with the aid of a long narrow forcep and then placed on already swabbed petri plates onequal distance .The principle for microbial inhibition procedures is the presence of clear zones on an agar plate medium to which bacterial spores have been seeded.

### 3.4.7.Statistical analysis

Data on any one area was inserted into Statistical Package for Social science (SPSS) version 16.0. Chi- square test was performed for comparison between different localities , between farms and markets and between cattle and sheep samples at 5% probability level to determine the percentage of antibiotic residues.

## Chapter four

## Results

Among the 50 milk samples collected from cattle and sheep (30 farm samples and 20 market samples),only 3 samples (6%) tested clearly positive for antibiotic residues with apparent inhibitions zones (as indicated in tables (4. 1 ,4. 2 and 4.3)and Figure 4.1.

A Farm sample gathered from Al-rodowan gleaner situated within Omdurman locality formed a clear circular inhibition zone measuring about 34 mm around the milk containing well and 39mm zone around the milk- impregnated whatman's filter paper . The other 2 sampleswere bought from the markets in the same locality produced irregularly shaped inhibition zones and only around the well measuring 15 mm and 8 mm in width while 21 mm and 23 mm in length ,respectively .

**Table (4.1).Percentage of positive milk samples collected from 4 locations in Sudan.**

| Antibiotic residues | Localition | | | | Sig Level |
|---|---|---|---|---|---|
| | East Nile | Al Khartoum | Omdurman | Al gazeera | |
| Negative sample % | 26<br>52.0% | 3<br>6.0% | 8<br>16.0% | 10<br>20.0% | .010 |
| Positive sample % | 0<br>.0% | 0<br>.0% | 3<br>6.0% | 0<br>.0% | |

**(4.2).Percentage of positive milk samples collected from farms and markets in Sudan(November ,2017).**

| Antibiotic Residues | Source of the sample | | Sig level |
|---|---|---|---|
| | Farm milk | Market milk | |

| | | | | |
|---|---|---|---|---|
| Negative sample % | Count | 29 | 18 | .331 |
| | % of Total | 58.0% | 36.0% | |
| Positive sample % | Count | 1 | 2 | |
| | % of Total | 2.0% | 4.0% | |

**Table (4.3).Percentage of positive milk samples collected from sheep and cattle in Sudan.**

| Antibiotic Residues | | Animal species | | Sig level |
|---|---|---|---|---|
| | | Sheep | Cattle | |
| Negative sample % | Count | 3 | 44 | .652 |
| | %of Total | 6.0% | 88.0% | |
| Positive sample % | Count | 0 | 3 | |
| | %of Total | .0% | 6.0% | |

**4.1.Omdurman locality:**

Among the 11 cattle milk samples collected from Omdurman locality (as 4 farm samples and 7 market/tank samples);3 samples tested positives for antibiotic residues(27.3%) and out of these only one sample originated from farm milk as explained in table 4.4and 4.5.

**Table (4.4).Percentage of positive milksamples collected from farms and markets in Omdurman locality(November ,2017).**

| | Farm samples | Market samples | Sig level |
|---|---|---|---|
| No of samples | 4 (100%) | 7(100%) | .898 |
| Positive% | 1(25%) | 2(28.6%) | |
| Negative % | 3(75%) | 5(71.4%) | |

**Table (4.5).Percentage of positive milksamples collected from cattle and sheep in Omdurman locality (November,2017).**

| | cattle samples | Sheep samples | Sig level |
|---|---|---|---|
| No of samples | 11(100%) | 0 | —— |
| Positive % | 3(27.3%) | 0 | |
| Negative% | 8(72.7%) | 0 | |

**4.2.East Nile locality:**

Out of 26 farm samples(3 sheep and 23 cattle )examined from East Nile area (10 sample from Al-selait agricultural scheme,10 samples from Mahalab etnain and 6 samples from Al-eesailab area) ;none of them reacted positively for antibiotic residues as shown in table 4.6 and 4.7.

**Table (4.6).Percentage of positive milk samples collected from farms and markets in East Nile area(November 2017).**

| | Farm samples | Market samples | Sig level |
|---|---|---|---|
| No of samples | 26(100%) | 0 | |
| Positive % | 0(0%) | 0(0%) | ___ |
| Negative% | 0 | 0 | |

**Table (4.7).Percentage of positive milk samples collected from cattle and sheep in East Nile area(November ,2017).**

| | cattle samples | Sheep samples | Sig level |
|---|---|---|---|
| No of samples | 23(88.5%) | 3(11.5%) | |
| Positive % | 0(0%) | 0(0%) | ___ |
| Negative % | 0 | 0 | |

**4.3.Al-khartoum locality:**

Only 3 samples were collected fromthe markets only in Al-khartoum locality(Algeraif garb area) and were all found to be negative for the antibiotic residues as clarified in table 4.8 and 4.9.

**Table (4.8).Percentage of positive milk samples collected from farms and markets in Khartoum locality (November ,2017).**

| | Farm samples | Market samples | Sig level |
|---|---|---|---|
| No of samples | 0 | 3(100%) | |
| Positive% | 0 | 0(0%) | |
| Negative % | 0 | 0 | —— |

**Table (4.9).Percentage of positive milk samples collected from cattle and sheep in Khartoum locality(November ,2017).**

| | cattle samples | Sheep samples | Sig level |
|---|---|---|---|
| No of samples | 3(100%) | 0 | |
| Positive% | 0(0%) | 0 | —— |
| Negative % | 0 | 0 | |

### 4.4.Al-jazeera state:

Ten milk cattle sample were collected from only the market at Al-jazeera state .No positive result were detected as illustrated in table (4.10) and (4.11).

**Table(4.10).Percentage of positive milk samples collected from farms and markets in Aljazeera state (November ,2017).**

| | Farm samples | Market samples | Sig level |
|---|---|---|---|
| No of samples | 0 | 10(100%) | |
| Positive % | 0(0%) | 0(0%) | — |
| Negative % | 0 | 0 | |

**Table (4.11).Percentage of positivemilk samples collected from cattle and sheep in Al-jazeera state(November,2017).**

| | cattle samples | Sheep samples | Sig level |
|---|---|---|---|
| No of samples | 10(100%) | 0 | |
| Positive % | 0(0%) | 0 | — |
| Negative% | 0 | 0 | |

## Chapter Five

## Discussion

There is an increasing international concern about the extensive spread of antibiotics resistant globally.Presence of antibiotics residues in food of animalorigin such as milk ,milk products, meat…etc is one of the reasons contributing to this fastness. This study was conducted to detect antibiotics residues in cattle/sheep milk samples gathered from markets and farms in the different location of Sudan. There was positive detection with the percentage of 6%, 0.0%, 0.0% and 0.0% in Omdurman ,East Nile, Khartoum locality and Al-jazeera state respectively .

Many previous surveys were employed for the detection of residual antibiotics in the milk in the Khartoum state, (Omer, 2016) applied delvotest SP for the detection of antibiotics residues in 236 milk samples.He obtained about 21.18% positive results which is more higher than the results declaredin our study(table 4.1)and might be attributed to the sensitive techniques he used. (Elhassan, 2012)evaluated 64 milk samples for the presence of neomycin and tylosin and all collected samples tested positive (100%).Barakat (1995) detected antibiotics residues in 80 milk samples usingdelvotest P. He anounced that 8.75% which is nearly closed to our study(table 4.1) .Osman (2002) claimed that the percentage of positive samples for total samples examined was 0.8% and for the samples taken directly from the udder, it was 4.0%, while Mustafa (2001)searched for the antibiotics residues in 100 milk samples collected from different areas in Khartoum state and his results proved negative for all the sample investigated.

In Zimbabwe 73 samples of raw milk from 3 main dairy market board collection centers, were scanned for the presence of microbial growth inhibitory substances and 4.4% of the samples contained antibiotic residues (Chagonda and Ndiku wera, 1989).This result is lower than the results obtained in this study(table 4.1). On the other hand,in Lisbon 2248 samples of consumer milk were investigated in 1981 to 1985. Six

hundred and seventy four of them (30%) were found to include inhibitory substances .Which is comparatively much higher result. (Barbosa *et al.*,1991).

These differences might be due to the effect of seasons or type of test conducted. In Khartoum multiple factors affect the presence of antibiotics residues in milk such as wrong practices of milk sellers who add antibiotics to milk to avoid bacterial spoilage; when there are delays in milk marketing.Also the milkmen don't comply with the antibiotics withdrawal period when treating their animals as some may even milk these animals after treatment.

In Khartoum area, the 0% percentage may be due to the availability of high density of consumersthus it won't be necessary neither to add nor store the produced amount of milk for a longer period and transfer it to another area . The overall positive percentage in this study was low and this might be due to the very low number of samples collected in this area.

Moreover, 0% result of the samples tested from the Al-jazeera state might be due to the fact that the cattle owners who sells the milk in Soba area in Al-khartoum locality,milks the cows and travels to the Al-khartoum in the early cold morning hours(World Meteorological Organization);therefore adding the antibiotics to milk wasn't in need. While,In the East Nile localitythe 0% might be attributed to the fact that the milk wasn't being transferred for far away consumers and was sold only locally; thus addition of the antibiotics weren't a demand.This study proved a higher percentage of positive sample in milk gathered from markets(4% as indicated in table 4.2) rather than that collected at farms directly (2% asexplained in table 4.2)and in particularly that from Omdurman locality which is in part is elucidated through the mixing of

milk in Large milk tanks/cisterns in which the milk comes from different farms/sources.

The higher result in cattle rather than sheep is attributed to their larger number in the farms from which the samples were collected and that sheep's milk isn't sold at the markets for human consumption.

Nevertheless,the overall percentage of positive sample is deemed to be low owing to the fact of small sample size and that the method carried out for residue detection in this study is much less sensitive than the more recent commercially available test neither was itpreceded by anothermore sensitive specific test/s.

# Chapter six

# Conclusion and recommendations

## 6.1.Conclusion

This study expresses the relatively higher prevalence and level of antibiotic residues in raw milk of markets and small-scale dairy farms in Omdurman locality mainly and only .Absence of proper management, non-compliance to drugs withdrawal period as the major contributing factors to the occurrence of antibiotic residues in milk.From the above findings:

There is a low level of awareness that consumption of raw milk contaminated with antibiotic residues can predispose the consumers to health hazards and some practices along the milk value chain predisposed milk to contamination with antibiotic residues.The finding of the abusive level ofresidues(the relatively high percentage taking into account the small sample size achieved in this study) provides alarming situation on the use of veterinary drugs by most markets in Omdurman locality and Khartoum state at large.Inadequate technical infrastructure - in terms of conformity assessment system i.e food laboratories, inspectorate and control authorities, human and financial resources, national legislative and regulatory frameworks, enforcement capacity, management and coordination; decreases the ability to confront these challenges of monitoring and ensuring raw milk from not only small-scale dairy farmers but also milk from traditional farmers are safe and free from antibiotic residues.Such systemic defects may not only threaten public health but may also result in threatening food quality.

## 6.2. Recommendations

To guarantee the quality and safety of raw milk along the milk value chain it is recommended to:

- ✓ Practice good management.

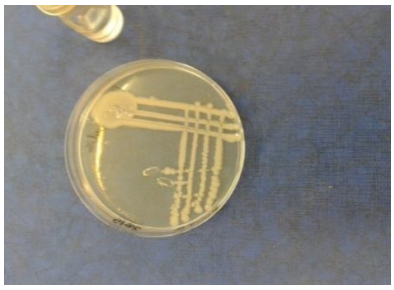
- ✓ build a valid veterinarian/client/patient relationship.
- ✓ Apply only FDA-approved, over-the-counter prescribed drugs with veterinarian guidance.
- ✓ Be certain to use only drugs that have labels that comply with state and/or federal labeling requirements.
- ✓ Store drugs properly.
- ✓ Administer all drugs correctly and identify all treated animals.
- ✓ Use and keep proper treatment records on all treated animals.
- ✓ Complete the milk and dairy beef residue prevention Protocol each year .
- ✓ The rapid antibiotic screening test i.e microbial susceptibility tests must be performed at the raw milk collection centres to ensure production of antibiotic residue-free milk as the initial step toward addressing the problem.
- ✓ Raising the awareness on the risks of consumption of raw milk contaminated with antibiotic residues amongst small-scale dairy farmers, rural and urban consumers. Nevertheless, key players and stakeholders in the milk sector like Veterinary and extension sectors, Food and Drugs Authority , Dairy Board, Milk Processing Association and Milk Producers Dairy Association should come-up with harmonized program and strategy to address this challenge of public health.
- ✓ Intervention at the farms level is significantly important because most of raw milk reaches consumers directly without processing stage.
- ✓ Socio-economic intervention should be enforced such as incentives to promote behavioral changes among small-scale dairy farmers that will enhance voluntary compliance of drug withdrawal periods.

- ✓ development of research in animal health and public policies focusing on the milk producers and dairy industry, for better quality of the milk produced.
- ✓ AQuestionnaire is suggested as a proper tool for acquiring significant amount of information regarding antibiotic residues.

## Appendixes:

## Appendix one

## Figures



**Figure(3.1)*Bacillus subtilis* subculture**

**Figure (3.2)Comparison between 0.5 Mac-forland solution and normalsaline-diluted colony**


**Figure (3.3)Clear inhibition zone in one of the positive samples in stage one.**

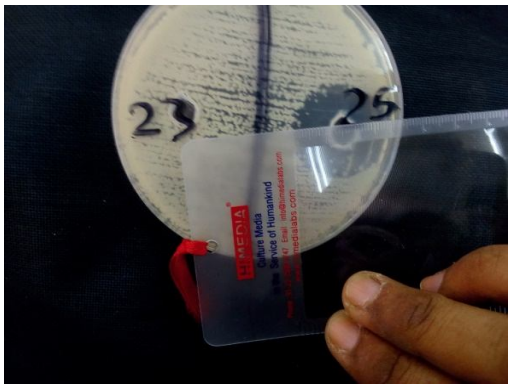



**Figure (3.4)Measuring of positive zone.**

**Figure (3.5)Note Sodium azide inhibits bacterial growth.**

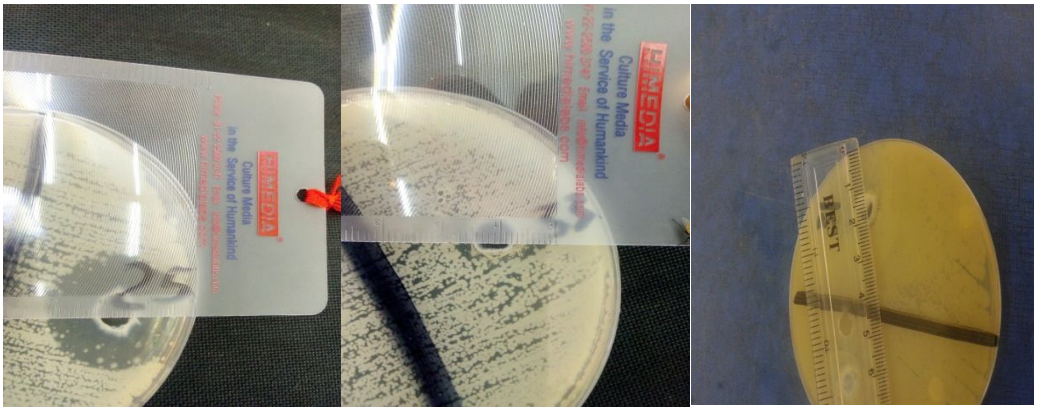
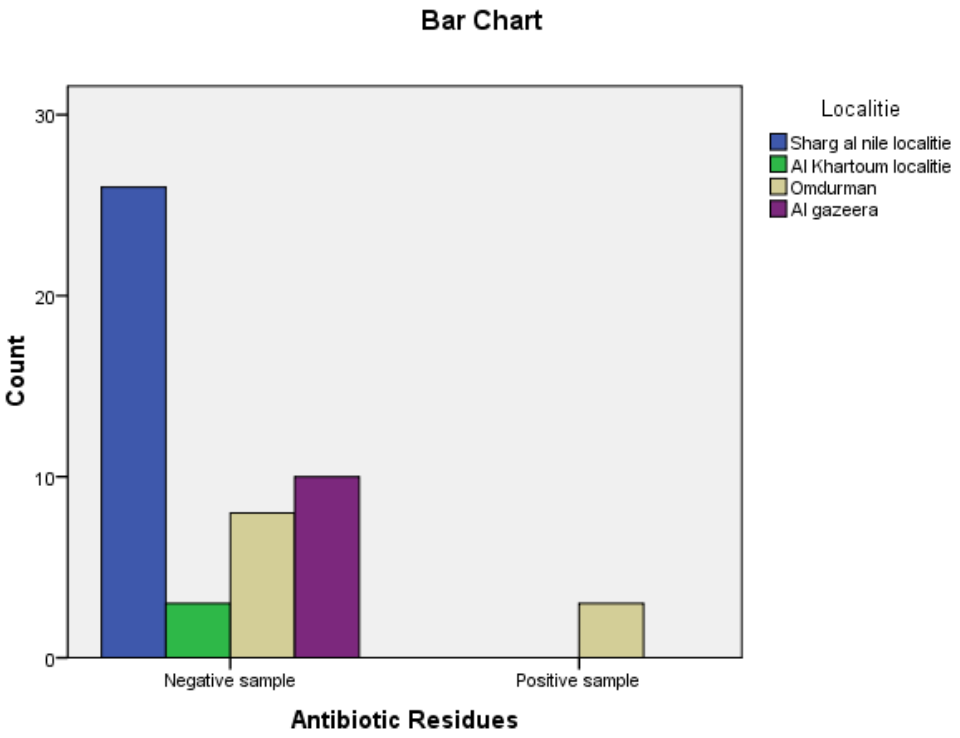
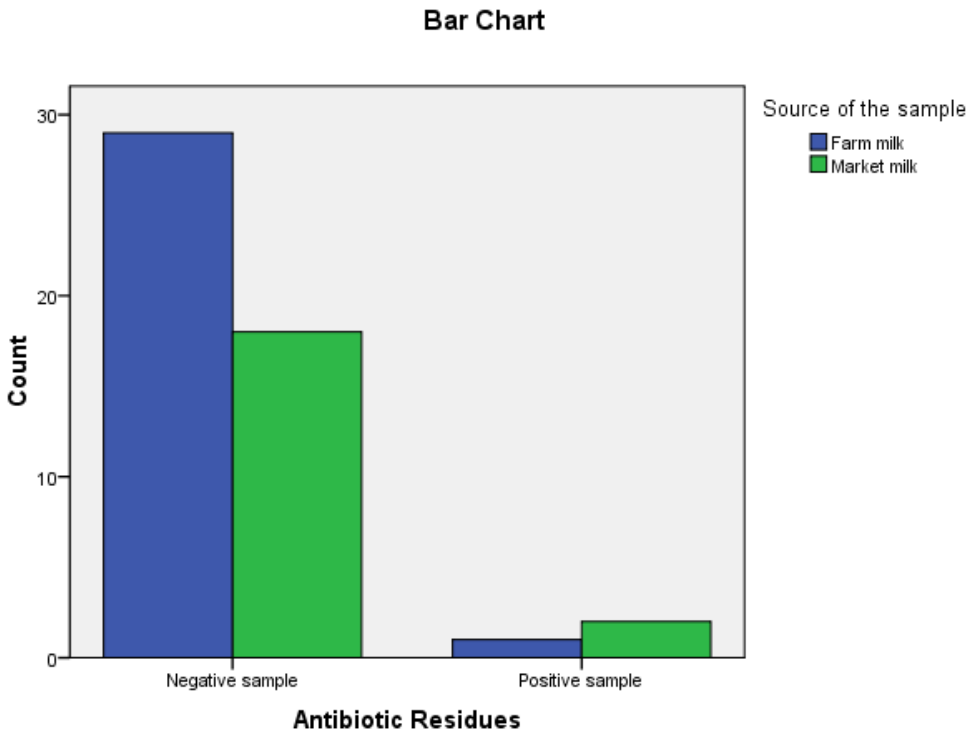



**Figure (4.1)Measurement of positive inhibition zones .**



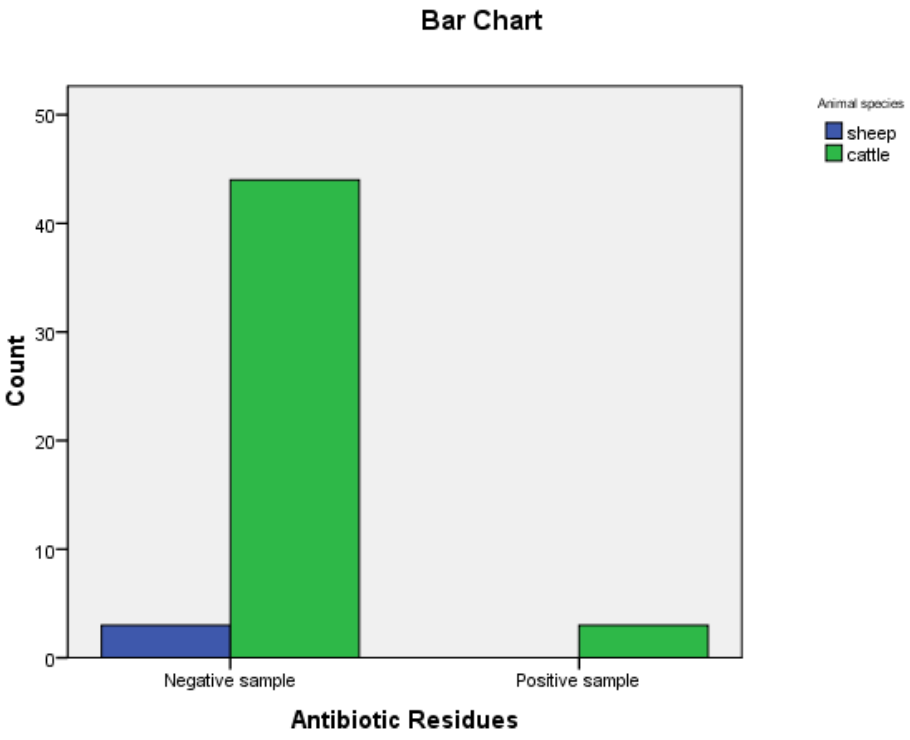

Source: Antibiotic residues survey in milk - Khartoum State Nov. 2017

**Figure(4.2)Percentage of positive and negative milk samples collected from four localities in Khartoum state.**




Source: Antibiotic residues survey in milk - Khartoum State Nov.2017

**Figure (4.3)Percentage of positive and negative milk samples collected from farms and markets in Khartoum state.**




Source: Antibiotic residues survey in milk - Khartoum State Nov.2017

**Figure (4.4)Percentage of positive and negative milk samples collected from sheep and goats in Khartoum state.**

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