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Sudan University of Science and Technology

College of Graduate Studies

Detection of Antibiotic Residues in cattle milk in River Nile state.

الكشف عن متبقيات المضادات الحيوية في لبن الأبقار بولاية نهر النيل .

Athesis submitted to the college of Graduate studies in partial fulfillment of the requirements for the Degree of Master in Preventive Veterinary Medicine (M.P.V.M)

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DEDICATION

With my love To:

my mother

my father

My brothers and sisters

My friend Najla Hassan

To all those who helped me, with all my best wishes

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ABSTRACT

This study was aimed to determining antibiotic residues contamination and the presence of penicillin and oxytetracycline in dairy milk ,in River Nile state.The study depending on previous survallence had been done since 2017 by department of veterinary public health and food safety incollaboration with Sudanese standards and metrology organization (Atbara branch).This study involved analysis of the data gathered in that survallance, all samples randomly collected from small-scale dairy farms ,local markets, selling milk and mobile venders in all six localities, that tested by using Ballya test in Central Veterinary Research Laboratory (CVRL)in Atbara for the presence of antibiotics residues.Thirty seven(20%)samples were collected from Aldammarlocality,seventyeight(47.03)samplesfromAtbara,eighteen(9.7%)samplesfromShandi,twelve(6.5%)samplesfromAlAlmatama,towenty(10.8%)samples from Barabar and twenty(10.8%)samples from Abohamad locality.Chi- square test was used for comparison between different localities and between farms , markets and venders samples at 5% probability level , to determine the percentage of antibiotic residues. Out of the 185milk samples , seven(3,8%)samples were positive to antibiotic residues , asfollows 2 (28,6 %) samples found in Aldamar locality , 2 (28,6%) samples one of them from farm and the other was bought from venders in Atbara locality and 3 (42,8%) samples were bought from market and vender in Shandi locality with p-value <0.055..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 River Nile state.

ملخص البحث

هذه الدراسة قامت علي تقييم التلوث لبقايا المضادات الحيوية(البنسلين والاوكسي تترا سايكلين) فى اللبن بولاية نهر النيل في ستة محليات(الدامر ،عطبرة،شندي،المتمة،بربروابوحمد).الدراسة اعتمدت علي مسح سابق تم في سنة 2017م بواسطة إدارة الصحةالعامة البيطريه وسلامة الغذاء بالولاية بالتعاون مع الهيئة السودانية للمواصفات والمقاييس (فرع عطبرة) .شملت الدراسة في الفترة بين نوفمبر 2019 الي نوفمبر 2020م جمع وتحليل البيانات لذلك المسح.كل العينات جمعت عشوائيا من المزارع والاسواق والباعة التجولين داخل كل الستة محليات،التي تم فحصها بواسطة Ballya test في معمل ابحاث عطبرة المركذى.37عينة جمعت من محلية الدامر ،78 عينة من محلية عطبرة،18 عينة من محلية شندي،12عينة من محلية المتمة،20عينة من محلية بربرو20 عينة من محلية ابوحمد.هذه المتبقيات لها اثر سلبي علي صحة الانسان. اختبار مربع كاي استخدم للمقارنة بين امحليات المختلفة وبين العينات الماخوذة من المزارع والاسواق والباعة المتجولين باحتمالية 5% لتقييم نسب بقايا المضادات الحيوية.من ضمن 185 عينة لبن كانت النتيجة 7(3.8%) عينات موجبة.2 (28.6%)من مزارع محلية الدامر،2عينة(28.6%)واحدة من المزرعة وواحدة تم ئ(42.8%)اثنين منهاتم شراؤها من السوق وواحدة من الباعة المتجولين بمحلية شندي. هذه الدراسه توصى بمسوحات أخري للبقايا في مراكز تجميع الحليب وتقصي ممارسات إنتاج الحليب بين المزارع صغيرة النطاق .هذا سيوفر أساس جيد لتصميم ممارسات مناسبة وفعالة لإنتاج الحليب في المزارع صغيرة النطاق للتقليل من تلوث الحليب والمساعدة علي حماية المستهلك في الولاية.

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Introduction

Consumption of milk is necessary to our life that's because it's primary source of nutrition for young mammals before they are able to digest other types of food. It contains many of nutrient's(calcium , magnesium , phosphorus , proteins , vitamins (A C B12- riboflavin) , iodine , zinc**.**There are two types of milk consumption: natural source of nutrition for all infant mammals and a food product for human of all ages that's derived from other animals. So that healthy life comes from healthy animals in the farm (Allison, 1985).

The present study is concerned about the milk as a public health view, therefore when a cattle in afarm take the medicine by the owner or a veterinarian with lack of information about the dosage or withdrawal period time and the extensive use of the same drug, all these reasons lead to the chemical residues in the tissue, urine, fat and milk. It is known that the excretion of the drugs is done by these routes but there are left over or trace amount of drugs called residues (Paige.*et al*., 1997 Riviere and sundlof, 2009). These residues cause many risks in the public health in human and the animal lives due to extensive use such as: resistance of the microbe against drugs, hyper products and find their way into the food chain (Riviere,., 1991 Sundlof,. 1994).

The control of these residues can be calculated as maximum residue limits (MRLs) and acceptable daily intake (ADI) of the drug according to FDA, FAO, and Codex Almantaries (Fitzpatrick *etal.*, 1995).Veterinary drugs are used in livestock to treat diseases, maintain herd and flock health, promote growth, improve meat and milk quality and increasing yield, otherwise reducing production costs (Richard and Gustafson ,1990)**.** Some examples of the most used drugs in the field benzylepencillines, tetracyclines ,ivermectines, sulphonamides, tylosin, gentamycineand phenylbutasone , which given in different formation by many routes such as I/V, I/M, I/Mm,S/C, Intravaginal/ intrauterine , orally...etc to treat several diseases like mastitis, pneumonia,parasitic infection and diarrhea

.(Riviere1991).

Objective:

To evaluate antibiotic residues using Ballya test in 6 localities(Atbara-Aldamer –Shandi-Barbar-Almatama and Abohamad) in River Nile state.

CHAPTER ONE

Literature review

1.1. Livestock in River Nile state:

A livestock census in River Nile state, reported about 3575887 units animals), cattle are estimated at about 10940 head, Goats1730023 head, sheep1432180 head and camel 84724 head. (Databases of the Ministry of Agriculture, Livestock and Irrigation - RNS2019

1.2. Milk: Definitions:

Milk is defined as the physiological secretion of the mammary gland of mammals to provide nourishment for their young. Throughout history man has recognized the milk value and dairy products as food not only for the young but also for the adults (Nickerson, 1999).Milk is the most complete food for all mammals, and this is especially true during the early period of life until weaning. It supplies the body with protein, fat, carbohydrate, minerals and vitamins in a manner to suit the nutritional requirements of the body (Omer, 2006).The milk consumption in the state was estimated in 2018to be about 67368.353tons, although the actual production 127110.1 tons is produced in the state.

1.3. Definition of the term Antibiotics:

The term antibiotic meant any microbial product which inhibitor kill certainmicroorganism (Singleton, 1995).

Antibiotics may be responsible for certain allergic reaction in man , moreover repeated administration of even a small amount may lead to the development of resistant strains of bacteria and therapy well threaten people's health (Jones, 1999).

Antibiotic therapy has been widely employed in the treatment of diseases in farm animals .Assessment of consumer safety of using veterinary medicines in foodproducing animals requires comprehensive set of data on the nature and quantity ofresidues in edible tissues and their basic pharmacokinetic role .this information isrequired to characterize the type and level of possible dietary intake of residues.

1.4.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 its administration as a stearatesalt (Brander and Puch,1982).Gentamycin is rapidly absorbed and readily distributed in to 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).

1.5.Interaction of antibiotics:

1.5.1.Synergistic

Discribes when the combined effect of two o rmore 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).

1.5.2.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).

1.6.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 should emerges out of the body in an unchanged form.Filteration of the drug in the renal tubules is through water reabsorption. (Bird and Nayler,1971).

1.7General classification of antibiotics and chemotherapeutic agents

1.7.1 Chemical classification

Chemical structure is the result of collaborative research programinvolving research group in Great Britain and the United States during
the years 1943-1945. Martin 1991and Todar 2000) define the groupbased on structure. Each group has a structural component that defines the group .Smith, (1966)and Reilly (1977) classifications has been based onchemical structure and propuse of action as follows:-

1. Beta Lactams and other cell wall synthesis inhibitor(Gale, 1981) Penicillin and cephalosporin cause lossof viability and inhibits synthesis of the cell
2. Other cell wall inhibitor Bacitrein and vacomycin
3. Membrane active affecting permeability and lead toleakage of intracellular constituents e.g polymyxins
4. Agents inhibit microbial protein synthesis:-

(I)macrolides these agents have large ring structure
and cause reversible inhibition of proteins synthesis
(chloramphenicol tetracycline

(ii) aminoglycossides composed of amino-sugar linked by glycosidic
bonds to various bases. The agents bind to 30s ribosomal sub-unit and cause accumulation of protein synthetic intiation complexes.

5. DNA polymerase inhiitor (Rifampin) affect nucleic acid Metabolism DNA Gyrase inhibitor e.g Quinolones.
6.Folate
antiagonists(sulphonamide,trimethoprim)Antimetaboliteswhich
block specific step that are essential to micro-organsims.

1.7.2 functional classification

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

(i) Broad spectrum antibiotics: these effective against gram positive and gram negative (Ampenicillin and Tetracycline).

(ii) Narrow spectrum: mainly effective against gram positive(Penicillin and Macrolides).
(iii) Drugs active against aerobic gra –negative bacteria.

1.8Mode of action

Generally antibacterial agents can be divided into groups affecting the synthesis of:

1. Nucleic Acid
2. Protein.
3. The formation of the cell wall
4. Cell membrane.

1.8.1 Antibacterial Action

A. Bacterio static antibiotics

Brander and Pugh (1977) mentioned that all antibioticsare bacteriostatic in suitableconcentration and these produce stasisof bacterial growth in vitro; this means that Invivo, the bacteria are made susceptible to the body defence mechanisms: Sulphonamides,Tetracycline, Chloramphenicol and Erythromycin.

B. Bactericidal antibiotics

These produce actual death of the cell in vitro so whenused clinically they should produce their therapeutic effect withoutthe aid of body's defence mechanisms.These antibiotics include Penicillin, Streptomycin,Neomycin, Bactercin and Cephalosporins

1.9 Types of Antibiotics

1.9.1 Penicillins

These are one of the most important antimicrobial agents. Although many other antimicrobial agents have been introduced since the discovery of Penicillin it is still widely used as a major antibiotic in asmuch as new derivatives of the basic nucleus are beingintroduced everyyear (Mandell and Sande, 1980).Fleming in1929) discovered Penicillin accidentally. He named it Penicillin after the organism that caused the bacteria to undergo lysis on a culture contaminated with the mold belonging to the species Penicillium notatum.

Penicillins is extensive chemical and physiochemical studies particularlywith the aid of x-ray cry-stallography provided un equivocal of the fusedB-Lactam thiazolidine structure of Penicillin. (Clarke *et al,* 1949).

In Penicillin Pharmacokinetics most absorption of Penicillin given orally takes place in thestomach and upper small Intestine. Once absorbed Penicillin are rapidlydistributed through most tissues.

Some Penicillin are metabolized by Liver but the kidneys are primaryorgans for excretion of *penicillin.* Also they are excreted through themilk in small quantities.

Pharmacodynamic of Penicillin bind reversibly with enzymes called Penicillinbinding proteins (PBP) outside the bacterial *cytoplasmic* membrane.

Penicillin act by impairing the development of bacterial cell wallby interfering with transpeptidase enzymes responsible for the formationof the cross link between peptide-glycan strands.These enzymes are involved in cell wall synthesis and cell division and when this binding occurs it increases the internal osmotic pressure and reptures the cell.

Some bacteria produce beta-lactamase penicillinase which increases the bacteria's resistance by converting Penicillin to in active Penicillinic

acid. Some Penicillins are more resistant to beta-Lactamase penicillinasehydrolysis and are referred to as beta-Lactamase penicillinase resistant.

Penicillins are divided into:

1. Narrow-spectrum B-lactamase sensitive Penicillins

Active against many Gram positive and a limited number of gram negative, also susceptible to B-Lactamase hydrolysis. (Aiello and Mays,

1998). E.g phenoxymethyl Penicillin and phenethicillin.

2- Narrow –spectrum Resistant B-Lactamase

In this group Penicillins are not as active against many grampositive bacteria (Penicillin G) also all gram negative (Oxacillin,Cloxacillin)

3. Broad Spectrum B-Lactamase sensitive Penicillins Against gram positive and gram negative e.g ampicillin andamoxicillin (Aiello and May, 1998).

4. Broad Spectrum- Resistant – B-Lactamase Penicillins It is fully active Penicillins against a wide variety of resistant bacteria, withdrawal time varies from 10-30 days. Milk should bediscarded for aperiod of 2 days for amoxicillin and 3 days for procaine Penicillin G.

1.9.2 Cephalosporins

In 1948 professor Giuseppe Brotzu hypothesized that the relative sterility of sea-water coast of Sardinia was due to substanceproduced by certain bacteria. These substances, he thought, inhibited the growth of other organisms.

In confirmation of his theory Brontzu isolated afungus, cephalosporium a cremorium, from sea water of the coast of Sardinia and found that it inhibited the growth of a variety of gram-positive and gram-negative bacteria.

Similar to Penicillin in various respects (Alexander, 1985).Cephalosponins divided into 3 generations.

First generation: quite active against gram positive and moderatelyagainst gram negative and not effective against an aerobes as Penicillins e.g Cephalothin, cephapiran Second generation: Active against both gram positive and gram negative more over they are relatively resistant to B-Lactamase e.g cephachlor and cefoxin.

Third generation **:**they are moderately active against gram positive but active against a wide variety of gram negative bacteria they are usually

highly resistant against B-Lactamase enzymes (Aiello and May, 1998).

1.9.3 Tetracyclines

They are broad spectrum antibiotics there are three naturally occurring members of this group:

Oxytetracycline,Chlortetracycline and Dimethyl chlortetracycline.

Pharmacokinetics of Tetracyclines can be by distribute rapidly and extensively in the bodyand in some instances they penetrate into C.N.S. deposited irreversibly in the growing bones and in dentin.

Withdrawal time (5-28 days). Excretions via kidneys & Gastro-intestinal tract.

The pharmacodynamics of this drug is act by binding reversibly to bacterial 30 s ribosmes and inhibitprotein synthesis generally bacteriostatic but at high concentration theybecome bactericidal because the organisms seem to loose the Functionalintegrity of the Cytoplasmic membrane. (Aiello and Mays, 1998).

1.9.4 Chloramphenicol

Its a relatively simple natural nitrobenzene derivative with abitter taste.

The pharmacokinetics by absorption occurs rapidly from the upper GI tract & maximum blood level occur in 1-3 hours. About 40-60% of it is in plasma is reversibly bound to albumin, the free fraction readily diffuses into almost all tissues including brain.

The principal route of excretion is Renal it cause irreversible a plastic anemia. Unlike other antibacterial agents, chloramphenicol under goes extensive hepatic metabolism withdrawal time 2 weeks.

In Pharmacodynamic this drug is highly effective and well tolerated broad-spectrum. Chloramphenicol inhibts protein synthesis by binding to 50s sub unit of70s ribosome and impairing peptidyl transferase activity it is a bacteriostatic but at high concentration may be bactericidal for some species (Aielloand Mays, 1998).

1.9.5 Quinolones

These are synthetic antibiotics (Renold, 1989)

Pharmacokinetics By 1/V, 1/M, and S/C they penetrate all tissues well and quickly.Some quinolones are eliminated unchanged e.g ofloxacin., some arepartially metabolized e.g giprofloxacin and enrofloxacin and some are completely degraded. Metabolites are sometimes active.Major excretion through Renal Route, Biliary (Cipprofloxacin andNalidix acid).

Quinolones appear in milk of lactating animals often at high concentrations that persist for sometime.

Pharmacodynamic

The quinolones act by inhibiting the enzyme DNA gyrase that is responsible for the super coiling of DNA so that the DNA can twist ina number of chromosomal domains and seal around an RNA core. When DNA-gyrase is inhibited by quinolones a reduction in the super coilingoccurs with aconsequent disruption of the spatial arrangement of DNA.Qunolones are usually bactericidal.

1.9.6 Sulfonamides

Derivatives of sulfanilamide

Pharmaco-kinetics were absorbed from the gastrointestinal tract (Burtis and Ashwood, 1991).Once absorbed they were bound to protein mainly toalbumin. About 60-90 percent of bounded protein was distributed to all tissues. The metabolism of sulphonamide was shown via N- acetylation.The product of metabolism had no antimicrobial effect.Excretion by urine, Bile and Feaces

Pharmacodynamic Sulfonamides are structural analogs of Paramino Benzoic Acid (PABA) and competitively inhibit on enzymatic step. (Dihydropterate synthetase) during which PABA is corporated into the synthesis of diyhdrofolic acid (Folic acid). This result in suppression of protein synthesis impairment of metabolic processes and inhibition of growth and multiplication they are most effective in early stages of acute infections when organisms are multiplying (Aiello and May, 1998). Trimethoprim is antibiotic which was used to complete the effect of sulfonamide. It was found to inhibit the reduction of dihydrofolic acid to tetrahydrofolic acid Brooks 1995.

1.9.7 Macrolides Antibiotic

theyhave typical lactone ring in their structure (Tylosine andErythromycin).

In pharmacokinetic they become widely distributed in tissues and tend to be concentrated in the spleen, liver kidneys and particularly the lungs.They enter pleural and ascetic fluids but not the Cerebrospinal Fluid (C.S.F).They concentrated in the biles and milk the concentration of macrolides in milk is several times greater than in plasma especially in mastitis (Aiello& May, 1998).

Pharmacodynamic Interfere with protein synthesis by reversibly binding to the 50s sub unit of the ribosome they are bacteriostatic but at high concentration erythromycin is bactericidal (Aiello and Mays, 1998).

1.9.8 The polypeptide antibiotics

Polymyxin are polypeptide antibiotics produced by differentstrain of Bacillus polymxa including Bacitracin ,Neomycin and polymyxin (Alexander, 1985).

Pharmacokinetic

Ziv (1981) stated that polymyxin are minimally absorbed from mucus surfaces and mammary glands. Peak Plasma levels are reached 2 hours after parentral administration. They undergo renal elimination mostly as degradation.

In pharmacodynamic They are bactericidal; they interact strongly with phospholipids in bacterial cell membranes and radically disrupt their permeability and function.The polymyxins are more effective against gram negative than gram positive (Aiello & Mays, 1998).-Freely diffusible by intramuscular route. Not suitable for oral use (destroyed by gastric juices) ,Freely diffusible, partly absorbedwhen given orally.Well absorbed when given orally.}Poorly absorbed; must be givenintravenously or intramuscularlywell absorbed when given orally.Well absorbed when given orally;enterohepatic recirculationmaintains high blood level.Variable absorption by the oralroute; lactobionate salt giveseffective intravenous injection.Well absorbed when given orally.Absorption is incomplete by theoral route; low but continuouslevels are obtained by the intramuscular route.

Not absorbed by oral route; goodabsorption when given by intramuscular route.

1.9.9 Aminoglycosides

Defined as a group of compounds, aminoglycosides is bactericidal group and have abroad spectrum activity against G +ve &G-ve bacteria (Singelton, 1995). It includes Streptomycin, Neomycin- Framycetin, Gentamicin, Kanamycin and Tobramycin.Pyatkin and Kuvoshein (1980), stated that

Streptomycin wasobtained from *streptomycesgriseus.* Neomycin from *streptococcus Frachiae* (FAO, 1995).

Pharmacokinetics

Absorption after I/M injection site is rapid and nearly complete > 90% availability and peak blood levels are usually achieved within 30-90 minutes.Absorption after intra-peritoneal administration can produce

serious side effects. Intravenous injection can be intermittent or continuous however continuous infusions have high risk of toxicity(Aiello and Mays, 1998). Because of their polarity at physiologic PH, the aminoglycosides distribute into the extracellular fluid space with minimal penetration into most tissues except the kidneys and the endolymph of inner ear. Aminoglycosides are eliminated unchanged in the urine.

In pharmaco dynamic they are more effective against rapidly multiplying organismsand they affect and ultimately destroy bacteria by several mechanisms.They need a short contact with the bacteria to kill them. Their main siteof action is the membrane associated bacterial ribosome through which interferes with protein synthesis by attachment to 30s Ribosome subunits causing misreading of messenger RNA (Alexander, 1985 and Aielloand Mays, 1998).

Toxicity of Aminoglycosides includes ototoxicity, neuromuscular blockage and nephrotoxicity.

Aminoglycosides Residues

Gentamicin, kanamycin and Neomycin in cow milk was studiedafter intramamary administration by Moretain and Boisseau (1993). Bycylinder plate method they suggested, that the sensitivity was 0.15 mg/ml Neomycin and Kanamycin and 0.05 mg/ml for Gentamicin. The mean elimination periods ranged between 4 and13 milking periods the provisional maximum residue limits (MRLs) for Gentamicin, Neomycin and Kanamycin in milk and tissue (0.1-5 mgkg-1) was detected by Haasnoot *et al* (1999). Posyniak *et al* (2001) detected the gentamicin and neomycin residues in animal tissues by liquid chromatography method. Limits of detection were 0.05 mg/kg and 0.10mg/kg for Gentamicin and Neomycin respectively. The residue of

streptomycin & dihydrostreptomycin in meat developed by liquid chromatography electro spray mass spectromein (LC-ESI-MS) (Horie *et al,* 2002).

Gentamicin

It is a new basic pseudo-oligosaccharid antibiotic. It is discovered by Brander and Pugh 1963. It is amixture drived fromMicromonospora (WHO, 1995)purpurea. So the spelling ending in micinis to indicate that the source is not streptomyces.gentamicin is an antibiotic administered to patients suffering from potentially lifethreateningbacterial infections. It has a narrow therapeutic range andconstant monitoring is necessary due to the fact that excess dosage can cause kidney and auditory nerve damageChemically, gentamicin is exceptionally stable, and is used extensivelyin animal husbandry. It can be stored at elevated temperatures forextended periods-of-time without loss of biological activity. Gentamicin occurs in four optically active analogs as figure 3 here.Physical properties

It is a powder, white to buff in color readily soluble in waterand heat stable.

Pharmacokinetic is oral absorption is minimal and so for systemic use Gentamicin must be given by parenteral route. It is absorbed very fast from the area of injection into serum since already one hour later the higher concentration with an average of 3.7 mg/ml has been reached (Baltimore ;Mary Land, 1970). Some thirty percent of the administrate dose of gentamicin is bound by serum proteins and released as drug is excreted. It excreted almost entirely by glomerular filtration high concentrations of the active form they are found in the urine. Fifty to one hundred percent of gentamicin injected can be recovered unchanged within 24 hours from urine of patient with normal renal function. Little antibiotic enters the cerebrospinal fluid, prostate and eye. However concentrations of between one-half and one-third of serumlevels are found in milk, bronchial secretion and other body fluids.

The main effect is ototoxicity with vestibular function being most often damage; there is also some kidney damage of high

doses. Arare but serious side effect is respiratory paralysis due to neuromuscular blockage this can be treated by parenteral calcium or anticholinesterase agent such as neostigmine (Brander, *et., al* 1985).

1.10 Metabolism and excretion of antibiotics

Drugs were removed from the body in unchanged form or converted to other substances. These conversions took place in the , liver kidney, or intestinal epithelium. The kidney excreted the unchanged drugs or its metabolites. A constant proportion of drug was removed in a unit of time it is called exponential clearance.(Archimbault 1983).Parke (1968) reported that on the whole system, these enzymes do not participate in the body's metabolism and are relatively non specific. A good antibiotic should be excreted in unchanged form. The drug was filtered in renal tubule by reabsorption of water (Bird and Nayler, 1971).

1.11Uses of antibiotics in food producing animals

1.11.1 Therapeutic uses

To control infection caused by bacteria and to get rid of disease causing on long-term health effects (Dixon, Tennant and Kay, 1993).

1.11.2 Prophylactic agent

To prevent out breaks of disease in particular circumstances (Dixon *et al,* 1993).Antibiotic as growth promoters.Antibiotic was approved by FDA (1951) as feed additive for animals to aid growth. Antibiotics were mixed with feed at subtherapeutic concentrations to suppress the activity of some of natural bacteria in animal intestinal tract (Dixon *et al*, 1993).

1.12Factors affecting drug residues

. Hapke and Grahwit (1987) approved that the concentration of drug in animal tissues is directly correlated to the absorbed dose.

. The route of drug administration, intramusular and subcutaneous injection causes high concentration and persistence of drug residue at the site of injection (Standers *et al*, 1988).

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

. Drug formulation affecting residues Baggot, (1992) stated that the only preparation of drugs are delayed in clearance after local intramuscular injection.

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

. Katz and Brady (1993) stated that deposition is the reason forvarying concentration in different tissues, high concentration must be expected in excretory organs.

1.12.Residues:

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

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

1.12.1Marker 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).

1.12.3.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).

1.12.4.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).

1.12.5.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 anduse 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 dryperiods 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 th 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 foodanimals 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).

1.12.7.1.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 directtoxicity 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.Immunopathologicaleffects,Autoimmunity,Carcinogenicity due(Sulphamethazine,Oxytetracycline&Furazolidone),Mutagenicity,Nephropathy(Gentamicin),Hepatotoxicity,Reproductive disorders,Bone marrow toxicity (Chloramphenicol),Allergy (Penicillin)(Nisha ,2008)and goitrogenicity (Kinsella,2009)risks have also beenobserved.

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.

1.12.7.2 Hazard and Risk associated with Antibiotics residues in milk

There are two types of hazards relating to drug residues direct short term hazards and indirect long term hazards. (Seri,2013).

-Directand short term hazard :

Drugs used in food animals can affect the public health because of their secretion in edible animal tissues intrace amounts usually called residues. For example,oxytetracycline (Salehzadeh et al., 2006) and enrofloxacin residues (Salehzadeh et al., 2007) have been found above the maximum residual level in chicken tissues. Similarly, diclofenac residues werereported to be the

cause of vulture population decline in Pakistan (Oaks et al., 2004). Some drugs have the potential to produce toxic reactions in consumers directly; for example, clenbutarol caused illness in 135 peoples as a result of eating contaminated beef in Spain in 1990. Other types of drugs are able to produce allergic or hypersensitivity reactions. For example2B –lactam antibiotics can cause cutaneous eruptions, dermatitis, gastro-intestinal symptoms andanaphylaxis at very low doses. Such drugs include the penicillin and cephalosporin groups of antibiotics (Paige et al., 1997).

-Indirect and long term hazards :

Indirect and long term hazards include microbiological effects, carcinogenicity, reproductive effects and teratogenicity. Microbiological effects are one of the major health hazards in human beings. Antibiotic residues consumed along with edible tissues like milk, meat and eggs can produce resistance in bacterial populations in the consumers. This is one of the major reasons of therapeutic failures amongst such peoples. Certain drugs like 3-nitrofurans and nitroimidiazoles can cause cancer in human population. Similarly, some drugs can produce reproductive and teratogenic effects at very low doses consumed for a prolonged period of time. One such example is vaginal clear cell adenocarcinoma and benign structural abnormalities of uterus with diethylstilbesterol (Sundlof, 1994). Posssible health risk associated with Antibiotics residues in milk :

The possible adverce effects of antibiotic residues were reported to cause allergenic symptoms , disorders of intestinal flora and resistance of bacteria to antibiotic administered (Deiatowr,1983).

These hazards and health risk include:

- Allergenic effects :

Antibiotic residues had the capability to bind directly or indirectly to the protein of the final antigen (Deiatowr, 1983). Penicillin was a well-known example , the antibiotic or its metabolite penicillenic acid were also do bind to the amino acid lysine. The penicilloylprotein conjugates were allergens. Cross immune-reactions which were neumerous between penicillin and different degradation products. were also used to produce identical conjugates. Senitization and allergic reactions were characterized by skin rashes and other unpleasant symptoms might occur in people already sensitive to a specific antibiotic (davis , 1986).

- Disorders of intestinal flora :

Antibiotics resiues could affect the human intestinal flora and disturb it (archimbault ,1983). One of these disturbance was that the human intestines bacteria become resistant to antibiotics through prolonged consumption of law doses of antibiotic (fox and cameron ,1985).

- Toxicological effects :

Antibiotic residues did not cause acute toxic effects due to their low quantity (archimbault,1983). However scientific studies on toxicological risks were done for each substance . The joint FAO/WHO expert committee on food additives studied the relation between choloramphenicol and a plastic anemia and suggested that no alteration in incidence ofplastic anemia would occur due to choloramphenicol residues in food but it required more studies in this subject (WHO, 1995 A).

Studies in experimental animals for streptomycin and dihydrostreptomycin (WHO, 1995 B) showed that they were responsible for accumulation in the perilymph of the inner ear , renal damage , atoxia , anemia and impairment of hepatic function.

Many antibiotics caused acute toxicity to the host when administered in high dosage (Burtis *et . al.,* 1991). For example aminoglycosides could cause acute tubular necrosis when given in a dose more than 35 microgram per milliliter. Sulphonamides could produce crystalline aggregates in kidneys , ureters and bladder when given in a dose more than 125 microgram per milliliter.

- Occurance of antibiotics resistance of bacterial strains :

Since 1960s , public health officials and scientist world wide had tried to quantity the role of antibiotics used in animals in bacterial resistance to antibiotics used for therapeutic purposes to treat human diseases(bonner,1997). The antibiotics were reported by (lewis,1995) not to cause technically the resistance but allowed it to happen by creating a situation where an already existing variant could flourish. The use of antibiotic in livestock might lead to resistance toantibiotics..

1.13.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).

1.14.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.

1.15.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 inamounts

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 orexposed 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, O TC and CTC) (Commission Regulation 37/2010; Spisso *et al.*,2010).

Table(1.2).MRL for Some Veterinary Drugs Antimicrobials MRL (µg/l)

Antimicrobials	MRL (µg/l)
Teteracylin	100
Cholorocycline	100
Oxyteteracyline	100
Doxyteteracycline	100
Benzyl pencillin(procaine)	4
Ampicillin	4
Amoxicillin	4
Dicloxacillin	30
Streptomycin	200
Erthromycin	4
Gentamycin	200
Tylosin	100
Lincomycin	150
Monensin	2 Monensin
Sarafloxasin	100
Spectinomycin	200
Sulfamethazine25	25
Sulfadimethoxine25	25
Sulfamerazine25	25
Sulfathiazole25	25
Sulfamethoxazole 100	100
Sulfanilamide100	100

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

1.16.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.

1.16.1.Biological Methods

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

1.16.1.1.Microbiological methods

Silver man and 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 microbial growth inhibition assays; which is a simple, sensitive and broadly drug-detecting test system (Andrew, 2001).Microbial] growth inhibition methods make the benefit of a standard culture of the tested microorganism in liquid/solid medium (Heeschen,1993).e.g .Geobacillus stearo thermophilus var. calidolactis ,*Bacillus subtilis*, Bacillus megaterium, Sarcinalutea, Escherichia coli,Bacillus cereus var. mycoides or Streptococcus thermophilus. The analysed milk sample is applied on the agar surface either directly or with a paper disc (disc assay plate methods). In the course of incubation, the diffusion of the sample into the medium

takes place (the agar diffusion principle) and if the sample contains inhibitor agents, prohibition or total inhibition of the tested microorganism growth occurs.

Depending on the method used, the existence of inhibitor agents in the tested sample is indicated by the formation of aclear zone of inhibition around the disc (disc assay plate methods) or a change in the medium colour (Botsoglou and Fletouris 2001).

Microbial growth inhibition methods (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 microorganism Geobacillus (Bacillus) stearothermophilus var. calidolactis: BR-test/AS/BlueStar/6/7 (Enterotox Lab., Germany), CharmBlue Yellow Test (CharmSciencesInc., USA), Delvo test SP-NT (Gist-brocades BV, The Netherlands),CMT – Copanmilk test (Copan Italia,Italy), Eclipse 50 (Zeu- Inmunotec S.L., Spain).Geobacillus stearo thermophilus is a remarkable testing microorganism for its properties from which the most important, according to Katzand Siewierski (1995), are: theability of rapid growth at higher temperatures (64°C) and a high sensitivity to the β- lactam antibiotics . Commercially available microbial inhibitor assays play an important role in the integrated detection system. At present, many commercially produced microbial inhibitor tests are done simultaneously with selective rapidtests for milk screening in primary production, in dairy industry and in accredited laboratories (Suhren1995;Honkanen-Buzalski and Reybroeck 1997;Honkanen-Buzalski and Suhren 1999; Botsoglou and Fletouris 2001).The advantage of these methods 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 of samples (Mitchell *et al.*,1998).These methods have their disadvantages, however, that limit their use: they do not enable specific antibiotic identification, have limited detection levels for a series of antibiotics ,regarded as qualitative only and require 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 anti microbialagents, if present in milk in higher concentrations, can bring about false – positive results (Andrew 2001;

Kang and Kondo 2001; Kang*et al.*,2005). Commercially generated microbial inhibitor tests are delivered in the form of ampoules (mono tests)or in the form of micro-plates with a high number of testing cells. Apart from water bath or incubator, they do not

request a special laboratory equipment. To avoid subjective variations in the visual interpretation and to take the readings in an automated and more objective manner; some authors performing photometric measurements use the appropriate Wavelength (590 nm) and another wavelength as reference (650 nm) in ELISA reader (Althaus *et al.*,2003).

When performing microbial inhibitor tests, it is a must to meet the standards of good laboratory practice (protection against the contamination of the test), checking the pH value of the sample, observing carefully the correct temperature and the incubation period as specified by the producer's instructions and testing a positive as well as a negative control alongside with the sample. Some of the microbial inhibitor screening methods, in frequent use are, for example: Eclipse test,

Charm Cow side test, 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 inoculated with 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 0C for 18-24hours. Following

incubation the cultures examined for bacteria growth inhibition zone .Incasey 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).

1.16.1.2.Enzyme linked immune sorbant assay (ELISA)

ELISA is highly specific and easy to apply from simple extraction 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 antibiotics and cross reaction with metabolites and compound with similar structure prevents accurate identification .So confirmation test with mass spectroscopy or high performance liquid chromatography (HPLC) are requested(Patal and Bond, 1996)

1.16.2.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 asolution 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 sulpha methazine and penicillin in milk. They extracted the antibiotics through employing acteonitrite while thin layer, electrophoresis uses an agar medium seeded with microorganism.

1.16.3.Electrophoresis

High voltage electrophoresis bio-autography was executed for identification of sulpha methazine 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.

1.16.4. Effect of heating on antibitic residues in milk :

Hapke and Grahwit (1987) approved that, the concentration of drug in animal tissue isdirectly correlated to the absorbed dose.

The route of drug administration intramuscular or subcutaneous injection causes high concentration and persistence of drug residue at the site of injection (Standers *et.al.*, 1988). Sumano *et.al.*, 1990 concluded that, the drug clearance in healthy and diseased animal are not same . In diseased animals , residue could persist two or three time longer than in healthy animals. The influence of the drug residue level in tissues based on difference in absorption and deposition processes that vary between differentanimal species (Baggot 1992). The oily preparation of drugs are delayed in clearance after local intramuscular injection .The absence of inhibition zone around the penicillin disc from milk after boiling for 15 min. Explained that the penicillin antibiotic is affected by heat and denatured , but if mixed with milk , it may conjugate with milk protein and will not be denatured upon heating. The overall results of all experiments indicated that heating or boiling of milk will not cause disappearance of antibiotic residues and hence the risk of antibiotic contaminated milk exist even after boiling (Abdulrahma2001).

In addition, Abdulrahman, 2001 found that the absence of any effect of heating on tylosin ,and very slight influence on oxytetracycline and penicillin in milk . The study indicated that there was aprobability of antibiotic hazard from contaminated milk to humans even after boiling. High levels of natural inhibitors are present in mastitis milk and in colostrums and they can cause false positive resullts in the microbial growth inhibition assays (Kang and Kondo, 2001). Moreover , they found that false positive result in the Delvotest assay correlated with an increase in lactoferrin and lysozyme concentrations.The heat treatment is a fast , simple and inexpensive method to remove false positive results as it has no effect on positive samples containing drugs . They also added that heat treatment before screening tests is an effective way to reduse false positive results in the milk samples.

1.17.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 Commission2002).

This framework has participated in the harmonization 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 falsepositives(Mensah *et al*., 2014).

CHAPTER TOW
Materials and method

This study was conducted between november 2019tonovember 2020 depending on data collected from previous survallance which had been done between 7-30 december2017 as follows

2.1. Study Area

This study was conducted in, River Nile state

2.2.Study population:-

Dairy cattle in River Nile state

2.3.Study Design :-

The study design was a cross sectional study.

2.4.Sampling Method:-

Amultistage simple random sampling method was adopted for milk samples collection from the different localities

2.5 Samples collection

Raw milk samples were collected randomly in clean sterile bottles from dairy farms, milk sale points, markets and mobile venders in the study area during the period 7 - 30 December 2017 to examine the remnants of antimicrobial drugs.

All samples were transported under refrigeration to the Central Veterinary Research Laboratory (CVRL)in Atbara and stored under refrigeration**.**

2.6. Source of the samples

samples was taken from Farms,Markets and mpbile venders in six localiteise(Atbara-Aldammer – Shandi, Almatama,Barbar andAbohamad localities) inRiver Nile state as follows:

(137) samples were collected from farms(18samples) ,markets(14samples) and mobile venders (5samples)in Aldamar locality,.

(78) samples were collected from farms(24samples) ,markets(29samples) and mobile venders (25samples)in Atbarar locality.

(18) samples were collected from farms(6samples) ,markets(9samples) and mobile venders (3samples)in Shandi locality.

(12) samples were collected from farms(6samples) ,markets(5samples) and mobile venders(1samples) in Almatama locality.

(20) samples were collected from farms(5samples) ,markets(11samples) and mobile venders(4samples) in Barbar locality.

(20) samples were collected from farms(8samples) ,markets(10samples) and mobile venders (2 samples)in Abohamad locality.

-Laboratory assessmentwas made for all samples included**:**

.

2.7.Materials:-

Ballya test. =Plate-stips-pipetter,incubater**.**

2.8.Procedure of the test

-raw milk and test kit were put in room tempriture, then 200ml of milk was dropped into sample-well and mixed with the reagent suffitiontely for 5minutes.

-The test strip was inserted into the sample-well after 10 minutes,tgen result was read.

2.9.Interpretation of the test:-

Result were read out from the lower two thirds of the agar medium as follows :

- No clear line appear indicated presence of antibiotic residues

- aclear line appears indicated absence of antibiotic residues .

3.4.Statistical analysis:

Data on any one area was inserted into Statistical Package for Social science (SPSS) version 16.0. Descriptive statistical analysis was displayed in frequency distribution and cross tabulation tables.. Chi-square test was performed for comparison between different localities , between farms , markets and mobile venders at 5% probability level to determine the percentage of antibiotic residues.

CHAPTER THREE

Results

185cow milk samples collected from cattle (67 farm samples .78 market samplesand40mobile vender samples among 6 localiteise for antibiotic residues appearance.table (3.1)

Among the185 cow milk samples collected from cattle (67 farm samples and 78 market samplesand40 mobile venders samples),only 7 samples (3,8%) tested clearly positive for antibiotic residues (as indicated in tables (4. 1 ,4. 2 and 4.3) Univariate analysis by chi-square at ($p<0.05$) shows that , there is no significant difference between six localities (p-value0.055) . table (3.5).

787 cattle milk samples collected from Atbara locality); 1farm sampleand1mobile vender sample both of them were pencillin tested positives for antibiotic residues, table (3.6)

Among the 18 cattle milk samples collected from Shandi locality (as6 farm samples , 9 market and3 Vendersamples);3 samples tested positivesforantibiotic Rresidues table (3.7).

Table 3.1 : Count and location of samples colected

Locality	**Farms sample**	**Markets sample**	**Venders sample**	**total**
Aldammer	18	14	5	37
Atbara	24	29	25	78
Shandi	6	9	3	18
Almatama	6	5	1	12
Barbar	8	10	2	20
Abohamad	5	11	4	20
Total	67	78	40	185

Table 3.2 Interpretation of the test:-

Locality	+ve samples	-ve samples	Total
Aldammer	2	35	37
Atbara	2	76	78
Shandi	3	15	18
Almatama	0	12	12
Abohamad	0	20	20
Barbar	0	20	20
Total	7	178	185

Table (3.3).Percentage of positive and negative milk samples collected from6localiteise inRNS

Antibiotic residues	Localition						total	Sig Level
	Aldammer	Atbara	Shandi	Almatama	Abohamad	Barbar		
Negative sample %	35 18.9%	76 41.1%	15 8.1%	12 6.5%	20 10.8%	20 10.8%	178 96.2%	.055
Positive sample %	2 1.1%	2 1.1%	3 1.6%	0 0%	0 0%	0 0%	7 3.8%	
Total	37 20%	78 42.2%	18 9.7%	12 6.5%	20 10.8%	20 10.8%	185 100%	

Figer 3.1 **Percentage of positive and negative milk samples collected from6localiteise inRNS**

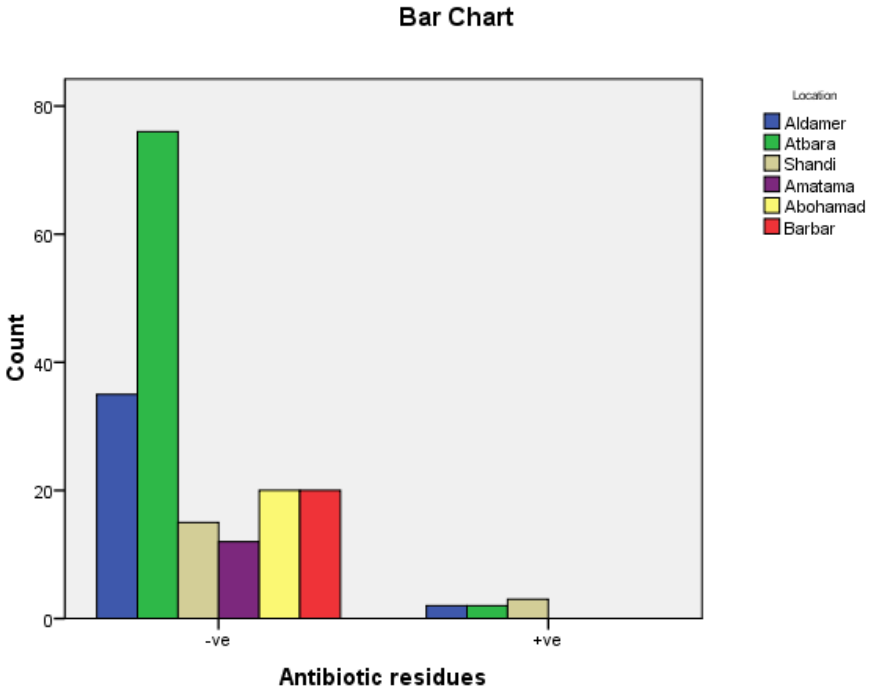

Table (3.4) Percentage of positive milk samples collected from3locations in RNS

Antibiotic Residue		Source of the sample		Sig level
	Farm samples	Market samples	Venders samples	
Negative Count	64	76	38	.752
sample % %of Total	34.6%	41.1%	20.5%	
Positive Count	3	2	2	
sample % % of Total	1.6%	1.1%	1.1%	

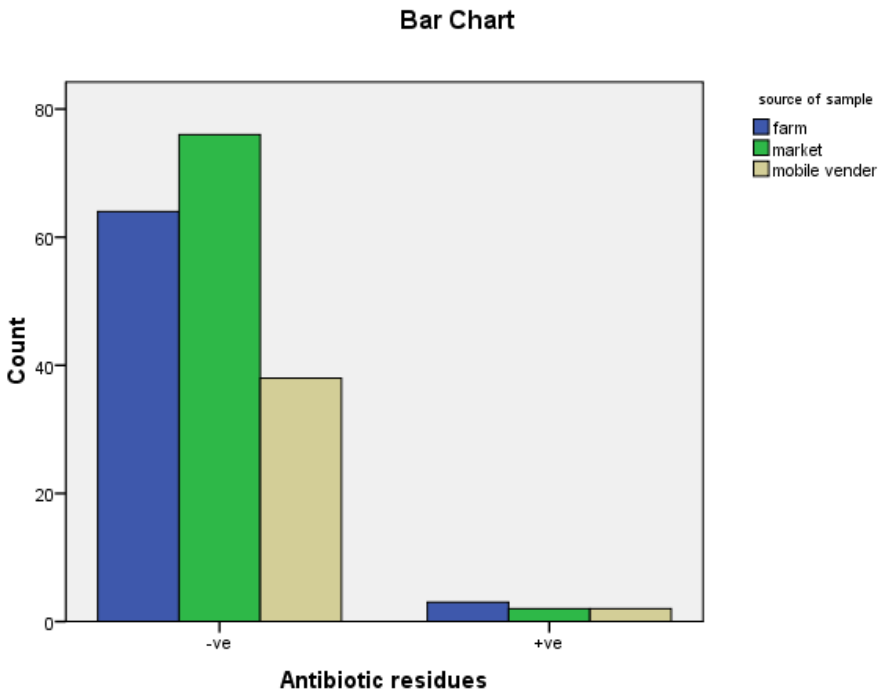

Fig (1) Anti biotic residues from6localiteise inRNS

Table 3.5. Percentage of positive milk samples Collected from farms,markets and mobile vender(1,1%in Aldammer**localit**

Result	Farm samples	Market samples	Vender samples	Sig level
+ve	2 5.1%	0 0%	0 0%	.292
-ve	16 41%	14 41%	5 12.8%	

Table (3.6).Percentage of positive milksamples collected from cattle1,1% in Atbara **locality .**

Result	Farm samples	Market samples	Vender samples	Sig level
+ve	1 1.3%	0 0%	1 1.3%	.544
-ve	23 29.5%	29 37.2%	24 30.8%	

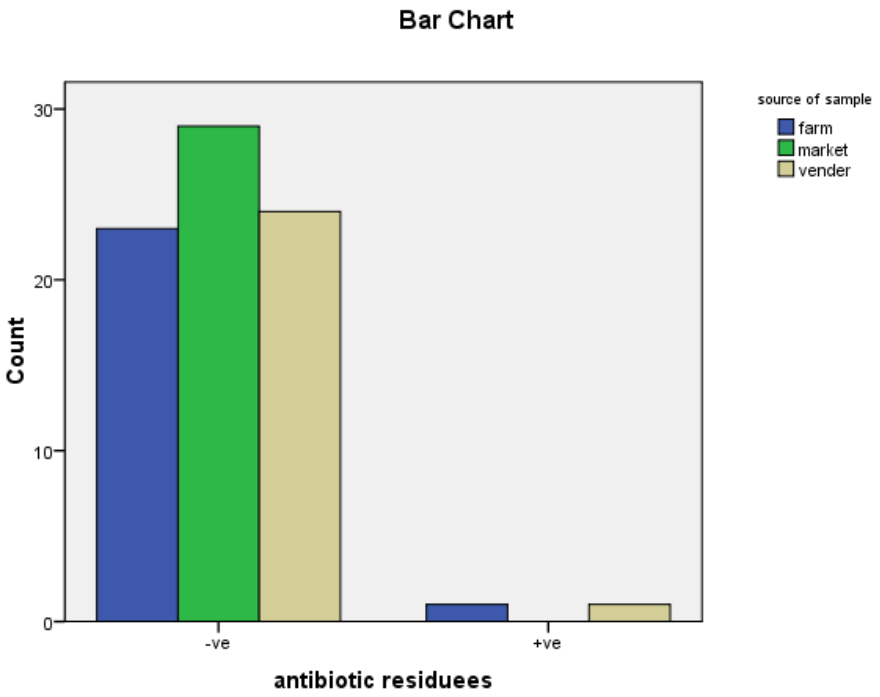

Fig (2) **Anti biotic residues in 3locations in RNS**

(3.7.Table.Percentage of positive milksamples collected from farms and markets1,6% in Shandi locality:

result	Farm samples	Market samples	Vender samples	Sig level
+ve	0 0%	2 11.1%	1 5.8%	.368
-ve	6 33.9%	7 38.9%	2 11.1%	

Table 3.8.percentageof antibiotic residuese in cattle milk from Atbara,Aldammer and Shandi localities in River Nile State :

Locality	Result	Farm sample	Market sample	Vender sample	Sig level
Atbara	+ve	1 1.3%	0 0%	1 1.3%	0.544
	─ve	23 29,5%	29 37.2%	24 30.8%	
Aldammer	+ve	2 5.1%	0 0%	0 0%	0.292
	─ve	16 41.0%	14 41.0%	5 12.8%	
Shandi	+ve	0 0%	2 11.1%	1 5.8%	0.368
	─ve	6 33.9%	7 38.9%	2 11.1%	

Table 3.9 Percentage of positive milksamples collected from farms .markets and venders0% in Amatamma locality

Result	Farm samples	Market samples	Vender samples	Sig level
+ve	0 0%	0 0%	0 0%	------
-ve	6 50%	5 41.7%	1 8.3%	

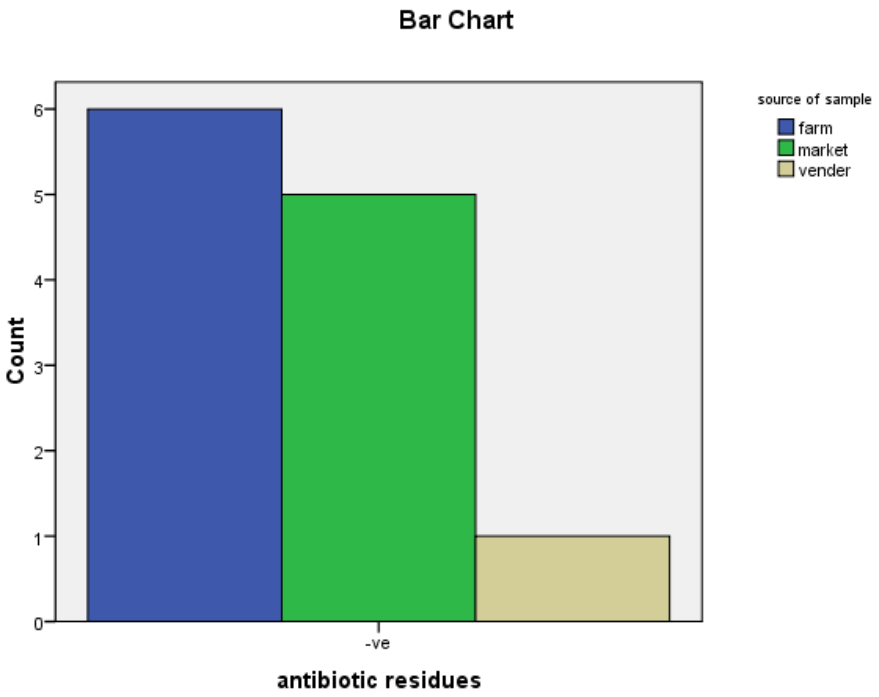

Fig (3) Anti biotic residues in Amatamma locality

Table 3.10.Percentage of positive milksamples collected from farms .markets and venders0% in Barbar l**ocality**

Result	Farm samples	Market samples	Vender samples	Sig level
+ve	0	0	0	---
-ve	8 40%	10 50%	2 10%	

Table 3.11Percentage of positive milksamples collected from farms .markets and venders0% in Abohamad locality .

Result	Farm samples	Market samples	Vender samples	Sig level
+ve	0	0	0	---
-ve	5 25$	1 55%	4 20%	

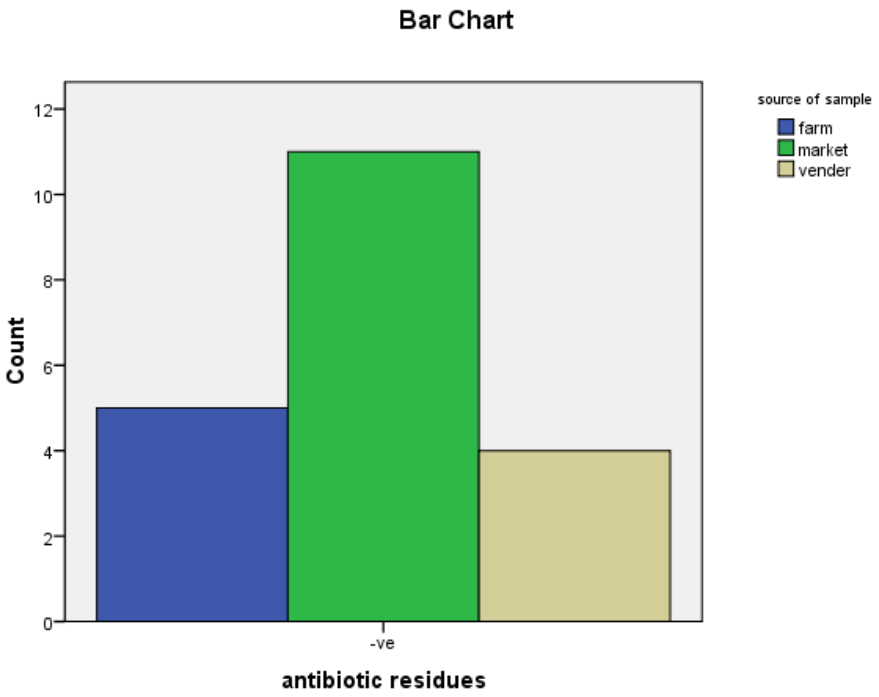

Fig (4) . Anti biotic residues in Abohamad locality

CHAPTER FOUR
DISCUSSION

There is serious international concern about the wide spread of antibiotics resistant at the global level. Among the reasons of this antibiotic fastness is the presence of antibiotics residues in animal's protein.

This study was conducted in River nile State, to detect antibiotics residues in milk collected from markets ,farms and venders sellers centre in the different localities of the state.Showed positive detection of 28.6%, 28.6% 42.8%,0%,0% and0% in Atbara,Aldammer, shandi,Almatama,Barbar andAbohamad localities respectively .Atbara and Aldammer are similler in peresentege of positive result but Shandi is the highest. This study proved a higher percentage of positive sample in milk gathered from farms(1,6%) (as indicated in table 3.2) rather than that collected at markets (1,09%)and venders(1,09%).

This is the first survey had done for the detection of residual antibiotics in the milk in River nile stat,While Many previous surveys conducted for the detection of residual antibiotics in the milk in Khartoum state, (Mona 2016) used delvotest SP for the detection of antibiotics residues in 236 milk samples, and she found that 21.18% gave positive results. This is by far higher than the results obtained in this study as this might bedue to the sensitive techniques she used. (Maha 2012) examined 64 milk samples the presence of neomycin and tylosin were detected in all collected samples positive (100%), Barakat (1995) used delvotest P for the detection of antibiotics residues in 80 milk samples. He found that 8.75% gave positive results, Raga (2002) stated 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) investigated antibiotics residues in 100 milk samples collected from different areas in Khartoum state where his results showed that all samples were negative. The percentage of this study was lowest than (Ammar2006) who reported that 38.9% of examined milk samples were positive by using Delvotest® SP-ampule kit , and (Tasneem,2006) found that 30.9% were positive to antibiotic

residues. Moreover, Adil *etal*2012) found that 33.1% of milk samples were positives by using also Delvotest® kit. Other studies in Indonesia found that 27.78% of samples were positive, Roostita *et.al.,.* Abdul Samad ,2014 in bakistan ,Sindh province observed that among the total of 400 samples of milk, about 49.75% were found to be positive for antibiotic residues.

In this study, River nile state many factors affect the presence of antibiotics residues in milk such as mal practice of milk venders who add antibiotics to milk to avoid the effectof bacteria, when there are delays in milk marketing.Also the milkers don't comply with the many antibiotics withdrawal time when they treat their animals as some any milk these animals in the same day of treatment. In Almatama ,Barbar and Abohamad localities , the 0% percentage may be due to the availability of consumers near milk production units or may be owing to the method carried out for residue detection in this study is much less sensitive than the more recent commercially available test neither was it preceded by another more sensitive specific tests .. .

Conclusion and recommendations

Conclusion:

The results showed that 3,8 % of milk samples tested in the sixth localities in River nile state was positive for antibiotic and the highest percentage of positive samples in milk gathered from farms . Farmers or cattle owners use antibiotics to prevent, treat and control diseases of their animals increase their productivity. 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.

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 abusivelevel 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 Shandi locality at large. However, there is a concern that routine antibiotics use in livestock management may have negative impact on human and animal health.

Recommendations

1. Education programs for farmer and milkers about usethe proper of antibiotics and observing the withdrawal period.

2. Regular checks for the residues of antibiotic in milk by veterinary authorities and qualified laboratories.build a valid veterinarian/client/patient relationship. The rapid antibiotic screening test i.e microbial susceptibility tests must be performed at the raw milk collection centres to ensureproduction of antibiotic residue-free milk as the initial step toward addressing the problem.

3.Raising the awareness on the risks of consumption of raw milkcontaminated 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.

4. Intervention at the farms level is significantly important because most of raw milk reaches consumers.

5. Sustainable veterinary supervision milk production in farms should be established through well- trained veterinarians .

6 .The proper choice of antibiotic screening test plays an important role in the effectiveness and accuracy of residue detection.

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