African Journal of Basic & Applied Sciences 6 (4): 87-97, 2014
ISSN 2079-2034

DOI: 10.5829/idosi.ajbas.2014.6.4.8642

Review on Detection of Antimicrobial Residues in Raw Bulk Milk in Dairy Farms

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Abstract: Antimicrobial are used for veterinary purposes almost as soon as they had been developed for human medicine. They are added to feed for growth promotion purposes, used for treatment of infections and are important during the development of intensive methods of animal husbandry (Dairy farms), as a result there are major hazards that could be posed by antibiotic residues in foods like raw bulk milk (such as allergic reactions, toxicity, carcinogenic effects, selection of resistant bacteria, disruption of human normal flora, provoke immunological response and inhibition of the starter culture). The risk of residue from the milk is higher in developing countries compared to developed one. This might be related with lack of facilities for detection and regulatory bodies that control the drug residues level in foods in the form of maximum residue limits (MRLs). Therefore, to prevent/minimize the risk of antimicrobial residue in milk, different methods of detection of residue to the standard limits level in bulk milk even in other food items is possible by chemical methods, microbiological methods and immunological assays. Even though, the sensitivity and specificity of these methods are different, they can provide either qualitative or quantitative data on antimicrobial (antibiotic) residue in milk. Generally Antimicrobial/antibiotics residue which is beyond the Maximum Residue Limit (MRL) can cause adverse effect on public health, dairy industry and Environment. The regulatory bodies should be formed and control the antimicrobial residue level in bulk milk before consumption (screening and quantitative evaluation of the level of antibiotic residue in milk). Sampling and testing protocols should be designed and properly applied.

Key words: Antimicrobial · Residue · Bulk Milk · Detection Methods · Maximum Residue Level

INTRODUCTION

Antibiotics have been used in the dairy industry for more than five decades in dairy cattle production to treat or prevent disease and to increase milk production or improve feed efficiency [1]. Residual antibiotics in milk can seriously affect consumers' health causing allergic reactions and developing resistant strains. Antibiotic contamination in milk can also cause significant economic losses for producers and manufacturers of milk and milk products. Although antimicrobial drugs are useful for treatment of human infections, their occurrence in milk causes adverse public health effects such as drug resistance and hypersensitivity that could be life threatening [2].

The use of antibiotics therapy to treat and prevent udder infections in cows is a key component of mastitis control in many countries. Due to the widespread use of antibiotic for treatment of mastitis in dairy cows, much effort and concerns have been directed towards the proper management and monitoring of antibiotics usage in treatments in order to prevent contamination of raw milk. As widespread use of antibiotics has created potential residue problems in milk and milk products that are consumed by the general public. Because of the public health significance, milk and milk products contaminated with antibiotics beyond a given residue levels, are considered unfit for human consumption [3]. The good quality of milk must contain no harmful or toxic residues, such as antimicrobial drugs. The extra-label use of these antimicrobial, insufficient withdrawal period and lack of records are the most common causes of theses residue in milk, which lead to exceed these residues in milk above the acceptable maximum residue limits (MRLs). In addition the lack of good veterinary practice and illegal use of veterinary drugs by farmers will increase this problem [4, 5].

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To detect antibiotic residues in milk different methods were developed and are applied in laboratory analysis. These consist of screening and chromatographic techniques to detect as many antibiotics as possible. The screening method is generally performed by microbiological, enzymatic and immunological methods. The screening methods are based on various susceptibilities of bacteria to different antibiotics. The antibiotic residue detection assays that are currently available use different methods and test microorganisms [6]. Microbiological assays for the detection of antibiotic residues utilize bacteria such as *Bacillus stearothermophilus* because of its high sensitivity to the majority of antibiotics. Both microbiological and chromatographic methods have been described for monitoring antibiotics in milk and animal tissues. Although the microbiological assay techniques have been recommended as official and conventional methods because of their simplicity, the bioassay methods lack specificity and provide only semi-quantitative measurements of residues detected and sometimes produce false positives [7]. Therefore, chromatographic techniques, such as thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), have been developed to replace microbiological assays [8].

In order to safeguard human health, the World Health Organization (WHO) and the Food Agriculture Organization (FAO) have set standards for acceptable daily intake and MRLs in foods [9]. Regulatory limits for antibiotic residues have been imposed on the dairy industry in many countries [10, 11].

Definition and History of Antibiotics: Antibiotics are substances either produced by one microorganism or produced synthetically or semi-synthetically that inhibit the growth of other microorganisms. Antibiotics are a subdivision of antimicrobial [12].

Antibacterial chemotherapy developed by Paul Ehrlich at the end of the 19th century was limited to the treatment of syphilis. It was followed by modern antibiotic development with a chance observation by Fleming in 1928 of a contaminating penicillium that produces a substance that inhibits *Staphylococci* growth. Consequent studies by Florey, Chain and other researchers resulted in the isolation and characterization of the pure, active constituent, penicillin [12].

In the 1940s and 1950s, investigations in both Europe and the United States into antibiotic substances produced from molds, *Streptomycetes* and other bacteria yielded many important antibiotics such as streptomycin, the original tetracycline, chloramphenicol, erythromycin, novobiocin and kanamycin, among others. By 1960, semi-synthetic β-lactam antibiotics of immense importance were produced by substituting various side-chains in the place of the original naturally occurring ones of the penicillin and cephalosporin nuclei [13].

Antibiotics are vital bioactive and chemotherapeutic groups of compounds made by microbiological synthesis usually used for the treatment or prevention of most types of diseases caused by infectious agents that are encountered in humans [14]. Moreover, in veterinary medicines, they are used to improve or maintain the health of animal species.

Antibiotics are administered to animals by several different routes, either injections, or orally in the feed and water, or topically on the skin or by intramammary and intrauterine infusions. The most commonly used antibiotics in feed animals can be grouped into five major classes. These include the beta-lactams (ß-lactams) (e.g. penicillins and cephalosporins), tetracyclines (e.g. oxytetracycline, tetracycline and chlortetracycline), aminoglycosides (e.g. streptomycin and gentamicin), macrolides (e.g. erythromycin) and sulfonamides (e.g. sulfamethazine) [15].

Classification of Antibiotics: Antibiotics are categorized according to their chemical structures (Table 1).

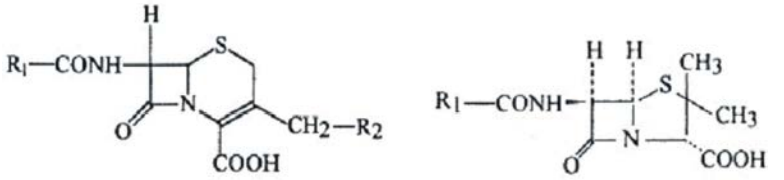
Beta-Lactam Antibiotics: The Beta-Lactams are the oldest and mostly used antibiotics among all others. Beta-lactam group of antibiotics are used especially to fight mastitis which is a serious disease that causes considerable economic losses in world's industry. Penicillins and cephalosporins both have beta- lactam ring where in the case of penicillins it is fused to a five-membered thiazolididine ring and in the case of cephalosporins it is fused to a six-membered dihydrothiazine ring (Figure 1) [17]. MRLs for beta-lactams are presented in Table 2.

MRLs for beta-lactams and other veterinary drugs have been set by the European Union for food producing animal. For example, the MRLs is 4 $\mu g\ kg^{-1}$ (4 ppb) for benzylpenicillin and ampicillin in milk. The food industry and the respective authorities carry out control programs and monitoring for drug residues in food for the good of public health and to avoid financial loss [17].

Table 1: Antibiotics classification according to chemical structure

Group	Internal group	Representative with practical importance
Carbohydrate antibiotics	1.Aminoglycosideantibiotics 2.Other(N- and C-) glycosides	Streptomycin, Neomycin
Macro cyclic lactone (lactam) antibiotics	1.Macrolide antibiotics	Erythromycin
	2.Polyeneantibiotics 3.Macrolactam antibiotics	Amphotericin
Quinone and similar antibiotics		Oligomycin
Amino acid Peptide antibiotics		Tetracyclines Penicillins, Cephalosporins, Bacitracin, Polymyxins
Nitrogen-containing Heterocyclic antibiotics		
Oxygen-containing Heterocyclic antibiotics	1.Non-condensed(single) heterocycles	No practical importance
	2.Condensed (fused) heterocycles	
	1.Furan derivatives	No practical imporance
	2.Pyran derivatives	
Alicyclic antibiotics	1.Cycloalkane derivatives	
	2.Small terpenes	Streptovitacins
	3.Oligoterpene antibiotics	
Aromatic antibiotics	1.Benzene compounds	Chloramphenicol
	2.Condensedaromatic comp.	Grisefulvin
	3.Non-benzene aromatic comp.	Novobiocin
Aliphatic antibiotics	1.Alkane derivatives	Varitin
	2.Aliphatic carbocyclic acid derivatives	

Source: Heeschen [16]



Cephalosporin Penicillin

Fig. 1: Structure of penicillin and cephalosporin (Source: Daeseleire *et al.*, [18]

Table 2: Maximum Residue Limits (MRL) for Beta-lactams

Antibiotics	MRL(ppb)
Penicillin G	4
Ampicillin	4
Amoxycillin	4
Cloxacillin	30
Dicloxacillin	30
Oxacillin	30

Source: [19]

Sulphonamides: The sulphonamide drugs that are used in animal production are soluble in polar solvents such as ethanol, acetone, acetonitrile and chloroform but insoluble in nonpolar solvents. This group has wide variety of polarity with amphoteric properties (pK 4.6- 11.5) due to the basic character of the para-NH2 group and due to N-H linkage adjacent to the sulphonyl group.

P-aminobenzenesulpone moiety is a part of many sulphonamides which reveals antimicrobial activity. In veterinary practice sulphonamides have been benefited as antibiotic agents in veterinary practice for several decades and are the fifth most widely used group in veterinary antibiotics in European Union countries, accounting to 2% of sales in 1997. Sulphonamides show antimicrobial activity with tri-methoprim, that is why they are frequently co-administered with this compound. Among many sulphonamides that have been defined, only few are approved for animals as veterinary medicine. The most frequently used sulphonamides are sulfadiazine, sulfadimidine, sulfamethoxazole, sulfadoxine and sulfadimethoxine. Within the EU, the MRL in milk has been determined to be 100 ppb. Some countries do not approve sulphonamides in food for human consumption and determination of sulphonamides requires methods that have low detection levels [20].

Sulphonamides like sulfamethazine (SMZ) and sulfadimethoxine (SDM) which are used improperly in lactating cows is a big concern. The residues of these antibiotics participate in milk which is an important component in the diets of young growing children and adults every day. Indeed, it was proved that SMZ is a

Table 3: Maximum Residue Limits (MRL) for Sulphonamides

Antibiotics	MRL(ppb)
Sulfamethazine	100
Sulfadimethoxine	100
Sulfamerazine	100
Sulfathiazole	100
Sulfamethoxazole	100
Sulfanilamide	100
Sulfadiazine	100

Source: [21]

Table 4: Maximum Residue Limits (MRL) for Tetracycline

Antibiotics	MRL(ppb)
Tetracycline	100
Chlorotetracycline	100
Oxytetracycline	100
Doxycycline	100

Source: [8]

potential carcinogen which raises major concerns Sulfamethazine is used therapeutically to treat infections, to control the spread of diseases as preservative, to expand feed fertility and to increase growth rate. The withdrawal time for SMZ is estimated to be 15 days [20]. The MRLs of some sulphonamides are given on Table 3.

Tetracycline: Tetracycline antibiotics are close derivatives of the polycyclic naphthacene carboximide. Some of them are product of bacteria called *Streptomyces,* whereas others are semi synthetic products. They are largely used all over the world as oral and parenteral medications and as feed additives for animals to promote food production as it active against both Gram-positive and Gram-negative bacteria [22].

Tetracyclines are used routinely in veterinary medicine for prevention and control of mastitis where they are re-added at sub-therapeutic levels to caddle feeds [8]. Only chlortetracycline and oxytetracycline are licensed among 10 antibiotic compounds as growth promoters for livestock in the USA. Tertracyclines have an extensive antibacterial spectrum and bacteriostatic activity. They also have a good activity against acute disease caused by Gram-positive and Gram-negative, which includes the species of *Spirochete, Actinomyces, Ricketsia* and *Mycoplahesma*. The use of these drugs against infectious diseases has become a critical problem, as their residues in milk or meat can be directly toxic or else cause allergic reactions in some hypersensitive individuals. Even more important, consuming of the food that includes low levels of tetracyclines for long periods can cause the spread of drug-resistant micro-organisms [8]. MRL's for tetracyclines are shown in Table 4.

Benefits of Antibiotics: Antibiotics are added to animal feed at low doses (less than 200 ppm) for two main reasons. Firstly, they are known to increase the growth rate and improve the feed utilization. Secondly, they are known to reduce mortality and morbidity from sub-clinical infections by preventing common animal diseases. How exactly antibiotics promote growth and increase feed efficiency is not well known [23].

Almost 90% of all antibiotics used in farm animals and poultry are reported to be administered at sub-therapeutic concentrations. About 70% of this is for the purpose of disease prevention and 30% are for growth promotion. Antibiotics have major effect in unsanitary environments. Their use controls the spread of infectious disease in crowded conditions. Diseases controlled by the usage of antibiotics include dysentery, mycoplasmosis and pneumonia. It is predicted that without the usage, the frequency of these diseases would dramatically increase [23].

Antibiotics as Veterinary Drugs: Veterinary medicines are mostly administered to animals in order to treat diseases, protect their health and as dietary supplement. Animal drug residues found in milk are a major health and regulatory concern. These drugs are mainly sulfa drugs, sulfamethazine and antibiotics known as penicillin and tetracycline.They are administered orally as feed additives or directly by injection. The use of antibiotics may result in drug residues in the milk, especially if not used according to label directions. The antibiotic residues in milk may cause allergic reactions in sensitive individuals, inhibit the growth of starter cultures in the production of cheese and other dairy products, or indicate that the milk may originate from an animal with a serious infection [24].

Antibiotic residues enter the milk supply chain at farm level. Therefore, it is important that producers realize the factors that lead to antibiotic residues in milk and how these residues can be avoided. Furthermore, the milk testing program should become a component of the quality control process centred on the farm, measuring the success of the industry in producing high quality milk and not being a regulatory program looking for flawed products [25].The usage of antibiotic varies from country to country, within a country and between farms, depending on policies. Moreover, the systems used to detect antibiotics in EU countries are developed and implemented by governments, companies and farmers exhibiting many differences [25].

Implications of Non-restrictive Uses of Antibiotics in Dairy Cows

Public Health Aspects: Human health problems that may result from intake of sub chronic exposure levels include allergic reactions in sensitive people, toxicity, carcinogenic effects [26] although the validity of some of the reactions is sometimes debated. Penicillins especially, as well as other ß-lactam antibiotics such as cephalosporins and carbapenems could cause allergies if high levels of residues persist in milk consumed by penicillin-allergic persons [27]. Tetracycline residues also have the potential to stain teeth of young children.

All antibiotics are capable of producing toxic effects, depending on the dose, the time of exposure and the mode of administration. To minimize human exposure to antibiotics from feed, prescribed withdrawal periods are required to be followed by the animal producers. No residues will remain in milk or meat if the required drug withdrawal schedule is followed. Following the withdrawal times guarantees the safety of milk and milk products for the consumer however may lead to economic losses for the farmer [28]. Withdrawal times may vary for particular drugs, dosage, duration and species. The extensive use of antibiotics led bacteria to develop defense mechanisms against antibiotics [29]. Residues in milk should be avoided since milk from treated cows may contain large number of potential pathogens and there might be biologically active metabolites or unchanged drugs in the milk causing an adverse effect to the consumer.

Antibiotic-resistant bacteria are transferred to humans by direct contact with animals fed with antibiotic containing feed or by persons harboring antibiotic-resistant bacteria [29].

Technological Aspects (On Dairy Processing Industry): The dairy starter cultures currently used in dairy industry for the primary acidification of the milk belong mainly to the genera *Lactococcus*, *Streptococcus*, *Leuconostoc* and *Lactobacillus*. These starter cultures are mainly lactic acid bacteria used in the production of a range of fermented milk products, including cheese, yoghurt, cultured butter and cultured milks. The primary role of starter cultures in cheese manufacture is the production of lactic acid from lactose at a consistent and controlled rate. The consequent decrease in pH affects a number of aspects of the cheese manufacturing process and ultimately cheese composition and quality [30].

Antibiotic residues in milk are undesirable from a manufacturing perspective, as they can interfere with starter culture activity and hence disrupt the manufacture process [30, 31]. Total inhibition of the starter culture has been observed to occur at approximately 60 µg/kg penicillin G. The sensitivity of starter cultures to antibiotic substances present in milk also varies considerably. Even within the same species of culture strain, differences in sensitivity are evident. Further, the response of starter cultures to residual antibiotics in milk destined for cheese or yoghurt manufacture can also be affected by the presence of other natural potential inhibitors [32].

Environmental Aspects: Active metabolites of antibiotics may be excreted by animals through urine and faeces and reach the soil and water. The most prevalent antibiotics found in the environment (surface waters) belong to the macrolide and the sulfonamide groups [33]. Tetracycline, penicillins or fluoroquinolones have only been found in some cases and at low concentrations [33, 34]. Zuccato *et al.* [35] identified some commonly used antibiotics, such as erythromycin, cyclophosphamide, sulfadimidin and tetracycline as antibiotics, which persist in the soil and remained in surface waters and soils for over a year.

Antibiotic metabolites have also been found to be able to be transformed back to their original active substances once in the environment [36]. Since most antibiotics are water-soluble, up to 90% of a dose can be excreted in urine and up to 75% in animal faeces. It has however been difficult to ascertain whether the residues are caused by waste water management or if they are due to inputs from agriculture. It is thus generally felt that dairy animals have a low influence on the input of antibiotics into the aquatic environment [33].

Sources of Contamination: The normal and predominant source of milk contamination with antibiotics is the intra-mammary application of the specific antibiotic, where untreated quarters may be contaminated via blood circulation or diffusion [23]. FDA surveys have shown that the main reason for residues in milk supply is the illegitimate use of drugs to treat mastitis in animals. Percutaneous, intrauterine, subcutaneous, intramuscular and intravenous applications of antibiotics are the other ways of secretory milk contamination. Contamination can also occur during milking where the inner surface of the parts of a milking machine are rinsed after milking of treated cows milk with untreated cows [23].

Detection of Antibiotics Residues in Milk: There are various chemical, microbiological and immunological assays used to detect antibiotic residues in milk. Among chemical methods are HPLC, gas-liquid chromatography, radioimmunoassay, TLC and electrophoresis [37].

Two steps are followed for the analysis to detect antimicrobial residues in milk. Firstly, an enzymatic or microbial or receptor-based method is used as screening tool. Second, the positive samples which contain antibiotic residues are confirmed by a chemical method. As a general rule, confirmatory analysis should identify the compound which is being investigated and to quantitate it. As a confirmatory method for antibiotic residues UV detector is used with high performance liquid chromatography. Because of its low sensitivity and selectivity many purification steps are needed to perform this method. Sometimes, to achieve higher sensitivity, a derivatization step is added to detect the analysts through a flourescence detector. The method takes long time because of purification steps and it is not adaptable for large number of samples.Highly sensitive and selective method can be applied to detect residues in milk with decreasing purification steps if liquid chromatography is coupled with mass spectrometry. Some methods that have been developed for antibiotic residue determination in milk does not exhibit enough sensitivity required by the tolerances set by the European Union Regulation 2377/ 90 [17].

The proper choice of antibiotic screening test plays an important role in the effectiveness and accuracy of residue detection. Screening tests are used to prevent the introduction of the contaminated milk into food chain and, therefore they are frequently used by regulators and food producers [19]. Screening tests can decrease the danger of residue contamination at violative levels if they are reliable to detect them at the concentrations found in bulk and tanker truck milk. Even though their performance is not understood so well, they still are important and necessary part of a farm total quality management program [38].

High Performance Liquid Chromatography in Residue Analysis (HPLC): HPLC is a separation technique that involves the injection of a small volume of liquid sample into a tube packed with tiny particles (3 to 5 micron (µm) in diameter called the stationary phase), where individual components of the sample are moved down the packed tube (column) with a liquid (mobile phase) forced through the column by high pressure delivered by a pump. These components are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. An output from this detector is called a "liquid chromatogram" [39].

High performance liquid chromatographyis now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples and industrial chemicals [39].

HPLC usage is increasing day by day in the field of residue analysis. The variety of mobile phases, the extensive library of column packings and the variation in modes of operations are the reasons for this method to be in demand. HPLC have progressed for determination analysis in food industry after all these advantages combined with various types of detectors available. In residue analysis of edible animal products, the sample often has much higher concentrations of endogenous interfering components but a very low content of residues. It is necessary to access variety of producers for isolations, derivatization and quantitation of the compound of interest since the nature and concentration of these components can vary widely [8].

Rapid Test Methods for Antibiotic Residues: Milk that contains antibiotic residues must be discarded. In the last years, the number of tests available has increased for detecting penicillin and other common antibiotics. There are some tests that are both qualitative and quantitative and some of them can be applied to detect antibiotics before they enter the milk supply at the source [40].

More purified and improved drugs are being used to treat cattle; because of this detection methods for residues are being refined and improved [40]. Rapid tests were designed in view of the needs of milk processors. These tests are simple and suitably sensitive and take very short time (10-20 minutes) to complete. The cause of the desire to shorten the duration times expedite the development of enzymatic and immuno/receptor tests. In the 1980's rapid detection tests were presented for the first time. These methods are more expensive than microbiological methods but their major insufficiency is that only materials that react with immobilized receptor can be detected, e.g., the beta-lactams [41].

Milk producers have to be sure that the milk they supply is free from the list of antibiotics that are prohibited, or that the levels of antibiotics are lower than MRLs. There is no microbial inhibitor test that can detect all substances at the MRLs set by European Union Regulations. Most of the methods are targeted to beta-lactam for the reason that they are most commonly used veterinary drugs in the therapy of cows in many countries. Enzymatic tests such as Penzym test, Penzym S (UCB Bioproducts, Belgium) and immunological tests such as Delvo-X-Press β-Lactam (DSM, Netherlands), β-STAR (UCB Bioproducts, Belgium), ROSA test (Charm Sciences, Inc., USA) are the most widely used rapid tests for antibiotic residues detection in milk [41].

Bacterial Growth Inhibition Methods: The inhibition of growth of responsive microorganisms was the mechanism of first methods to detect antibiotic residues in milk. A cyclinder plate assay method and filter paper disc method were used in the early 1940's. At first, *Bacillus subtilis* was used as responsive microorganisms but in recent years, methods have started to rely on *B. stearothermophilus* inhibition. These assays are specific for beta-lactams but most have been developed for penicillin detection. Delvotest SP (DSM, Netherlands), Copan Test (Copan, Italy), Charm Farm-960 Test (Charm Sciences, Inc., USA) are the most commonly used microbial inhibitor tests which use spores of *B. stearothermophilus* var. *Calidolactis*. The principle for this test is comparing clear zones on an agar plate medium to which bacterial spores have been seeded. Zones that belong to sample are compared with the zones of known amount of penicillin for quantitative determinations. Sensitivity and reproducibility of the method is affected by the depth of agar, where a thin layer is more sensitive than a thick layer [40]. Acid production during growth of *B. stearothermophilus var.* C*alidolactis* is utilized to develop commercial Delvotest SP (DSM, Netherlands). If inhibitors are absent, the bacteria grow and produce acid, a change is seen in the indicator. Test kits are available for individual as well as for multiple sample analyses. A commercially available test kit BR TEST AS detects a host of inhibitory substances. Agar diffusion and color reduction techniques are combined in this method using *B. Steathermophilus var. calidolactis* spores. During incubation, the metabolism of the bacteria is inhibited if drug residues are in the excess of the detection limit of the method. Test color remains blue if inhibitors are present whereas during incubation of inhibitor-free milk, oxidation-reduction reactions change the color to yellow. This test is appropriate for raw and pasteurized milks [29].

Competitive Binding Methods: Various test procedures are developed by Charm Sciences, Inc. (Malden, MA) to detect inhibitory substances in milk. The original test, Charm Test, has been recasted a few times over the years to make its sensitivity and accuracy better and expand its selectivity. Final action procedure was developed for assay of beta-lactams in milk in 1984. In this procedure the principle is that beta-lactam residues have a specific, irreversible propensity for enzyme sites on the cell wall of microorganisms, 14C-labeled [42]. Penicillin and *B. stearothermophilus* vegetative cells are used for this method. If penicillin is present in the sample, it competes to bind enzyme sites on the bacterial cell wall and more 14C-label remains free in the solution. Positive and negative controls are prepared before sample analysis and results of these controls are compared to the sample within 15 minutes. Charm II procedure are being used by many dairy laboratories where seven families of antimicrobial drugs can be screened. Necessary binding sites are procured by two different microorganisms for the seven drug families. Beta-lactam, tetracyclines, macrolides, streptomycin, chloramphenicol, novobiocin sulphonamides and can be counted as these families. Biologically active drugs are detected in about 8 minutes for one or two families or 15 minutes for all seven families. Reagents are in tablets and single tests can be performed easily. Sensitivity of this method is good for β-lactam antibiotics and all sulfa drugs in raw milk, milk powder and pasteurized milk. Figure 2 gives a basic idea for the principle of Charm II Assay test. The sample is incubated with a binding agent and a tracer which contains labeled version of the antibiotic to be detected. The antibiotic residue in milk competes with this labeled antibiotic for the receptors on binding agent. A scintillation counter measures the amount of tracer on the binding agent and compares with a control point [29].

The binding of DD-carboxypeptidase to beta-lactam antibiotics is utilized for another competitive binding method. The Penzym-test is a rapid enzymatic test to detect beta-lactam antibiotics. The principle of the test is that β-lactam antibiotics inhibit the activity of DD-carboxypeptidase which liberates D-alanine from an enzyme substrate. Color change is the proof for the

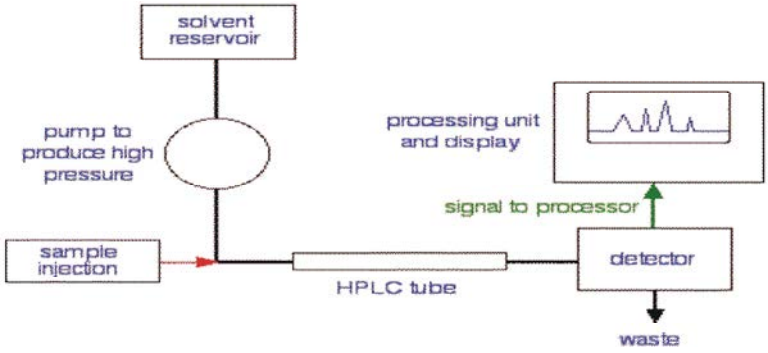


Fig. 2: A flow scheme for HPLC

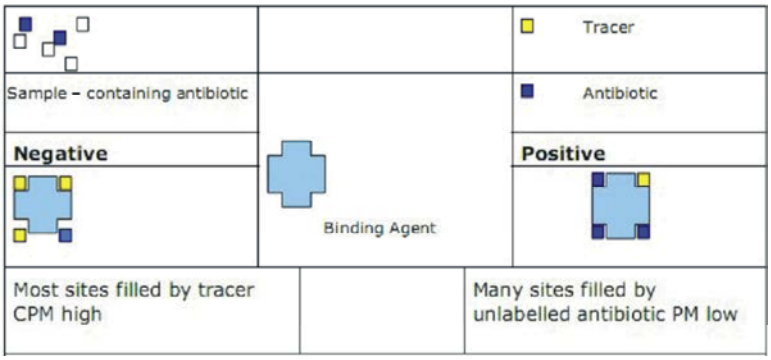


Fig. 3: Charm II Assay Test Procedure
Source: [42]

Table 5: CAC Residue Limits of common veterinary drugs (µg/kg) set for milk

Common Vet. Drugs	CAC MRL (µg/kg)
Benzylpenicillin/Procaine benzylpenicillin	4
Ceftiofur	100
Dihydrostreptomycin/Streptomycin	200
Diminazene	150
Febantel/Fenbendazole/Oxfendazole	100
Isometamidium	100
Neomycin	500
Oxytetracycline	100
Spectinomycin	200
Spiramycin	200
Sulfadimidine	25
Thiabendazole (used also as pesticide)	100

Source: [45]

antibiotic presence. If antibiotics exist in the sample, D-alanine cannot be liberated and no color change is observed. The test produces a yellow color if the sample is positive. This test is available in a kit and each of them should be checked before the use with penicillin standards, as the test detects beta-lactam residues at 0.01 IU/ml in raw milk. Positive and negative controls should be prepared for all samples. Results are ready in 20 min [43].

Maximum Residue Limits (MRL)

Definition of MRL: The maximum residue limit (MRL) is defined as the maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical that is recommended to be legally permitted or recognized as acceptable in or on a food, agricultural commodity, or animal feed. The concentration is expressed in milligrams per kilogram of the commodity (or milligrams per litre in the case of a liquid commodity) or ppm/ppb [44].

Regulatory levels have been established for drug residues in foods in the form of MRLs [26]. MRLs for veterinary drugs refer to the maximum concentration of a residue (resulting from the use of a veterinary drug) that is acceptable in food [45]. Sampling and testing protocols are based on standards set by CAC and Table 3 gives some examples of those, which have been set for milk from veterinary cows.

The MRL is based on the Acceptable Daily Intake (ADI) for a given compound, which is the amount of a substance that can be ingested daily over a life time without appreciable health risk. MRLs are fixed on the basis of relevant toxicological data including information on absorption, distribution, metabolism and excretion [46].

Table 6: Maximum Residues Limit (MRL) (ug/kg) for veterinary drugs

ANTIBIOTIC	MRL
Benzyl penicillin	4
Ampicillin	4
Amoxycillin	4
Oxacillin	30
Cloxacillin	30
Dicloxacillin	30
Tetracycline	100
Oxytetracycline	100
Chlortetracycline	100
Streptomycin	200
Dihydrostreptomycine	200
Gentamycine	200
Neomycin	100
Sulphonamides	100
Trimethoprime	50
Spiramycin	200
Tylosine	50
Erythromycine	40
Quinalones	75
Polymyxine	50
Ceftiofur	100
Cefquinome	20
Nitrofurans	0
Nitromidazoles	0
Other chemotherapeutics (Chloramphenicol, Novobiocin)	0

Source: [48]

The European Union (EU), through a regulation No. 508/1990 [47] has also set MRLs for antibiotics of which for the ß-lactams group includes, penicillin G 4 µg/kg, ampicillin 4 µg/kg, oxacillin 30 µg/kg, amoxicillin 4 µg/kg, dicloxacillin 30 µg/kg, cephalexin 100 µg/kg and cephapirin 60 µg/kg. MRLs are today assessed and established by the respective expert groups of different regions. In Kenya and most low-income countries, regulatory bodies do not present MRLs and only specify a zero tolerance. The specification is where no detectable residues are permissible in animal foodstuffs. This standard is not practiced internationally as they are no analytical techniques with the sensitivity to achieve it.

CONCLUSION AND RECOMMENDATION

The introduction of antimicrobial residue into milk chain can be mainly from dairy animals in which it is applied for treatment purpose, as food additives and as growth promoter. Generally Antimicrobial/antibiotics residue which is beyond the Maximum Residue Limit (MRL) can cause adverse effect on public health, dairy industry and Environment. The proper choice of antibiotic screening test plays an important role in the effectiveness and accuracy of residue detection. Screening tests are used to prevent the introduction of the contaminated milk into food chain and, therefore they are frequently used by regulators and food producers. Screening tests can decrease the danger of residue contamination at violative levels if they are reliable to detect them at the concentrations found in bulk and tanker truck milk. Even though their performance is not understood so well, they still are important and necessary part of a farm total quality management program. The regulatory bodies should be formed and control the antimicrobial residue level in bulk milk before consumption (screening and quantitative evaluation of the level of antibiotic residue in milk). Sampling and testing protocols should be designed and properly applied.

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