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REVIEW ARTICLE

Antibiotic Residues in Milk and Their Detection Methods: A Review

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Abstract

Milk and dairy products play a crucial role in human nutrition, but their quality can be affected by various factors, impacting marketability and consumption. Antibiotics are commonly used in the livestock industry for treatment and growth promotion. However, inadequate supervision and abuse by some producers, as well as improper drug use, can result in the excretion of drug and antibiotic residues into livestock products. These residues can then be secreted into milk and remain unchanged in the final product, potentially causing health problems for humans such as increased antibiotic resistance and hypersensitivity reactions. Therefore, it is essential to evaluate drug residues in milk and dairy products. Today, various methods are used to identify and quantify the amount of antibiotics in food. It is crucial for experts to choose a reliable and accessible method for this purpose. Implementing a dependable monitoring system, conducting regular inspections, and preventing excessive drug use are all vital steps in minimizing drug residues.

1. Introduction

Milk has a short shelf life because of its high nutritional content and the potential for contamination by various microorganisms like Staphylococcus, *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, *Campylobacter*, etc. Therefore, temperature control is crucial from the milking process to storage in the raw milk tank and pasteurization process (Fusco *et al.*, 2020). Various chemicals from agricultural, veterinary, and sanitary pollutants may also enter milk. These substances include veterinary drugs such as antibiotics, hormones, anthelmintic, pesticides, and more (Khaniki, 2007).

Antibiotics are compounds produced from synthetic, semi-synthetic, or natural substances (Ahmed *et al.*, 2017). These compounds are mainly used to treat diseases and as food supplements. Additionally, they are administered orally or by injection to enhance the growth and increase the productivity of animals. If antibiotics are not used correctly, drug residues may remain in milk and affect the consumer (Schenck and Callery, 1998). More than half of the global production of antibiotics is used for animals (Girmatsion *et al.*, 2021), which can lead to increased resistance in microbes. Antibiotic residues may remain after treatment, so it is necessary to ensure that food from treated animals does not contain levels exceeding the standard (Vercelli *et al.*, 2023). The presence of antibiotic residues in milk may lead to resistance in microbes, a weakened immune system, allergic reactions, and even tissue damage and neurological disorders (Vercelli *et al.*, 2023). Some possible antibiotics found in

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milk include aminoglycosides, beta-lactams, tetracycline, macrolides, chloramphenicol, florfenicol and others.

1.1. Aminoglycosides

Aminoglycoside antibiotics consist of an aminoglycoside and an aminocyclitol ring linked by a glycosidic bond. These antibiotics include gentamicin, neomycin, dihydrostreptomycin, and streptomycin, and are primarily used in food-producing animals. Residues of aminoglycoside antibiotics, if present in milk, can damage the kidneys, cranial nerves, and lead to hearing loss. The FDA has set the tolerable levels of dihydrostreptomycin and neomycin in milk at 125 and 150 ng/mL, respectively, and the level of concern for gentamicin in milk at 30 ng/mL (Schenck and Callery, 1998).

1.2. Beta-Lactams

Beta-lactam antibiotics, such as penicillins and cephalosporins, are a concern due to the potential for causing allergic reactions in some individuals. In the United States, there are strict legal requirements regarding beta-lactam residues in milk, with all milk being mandated to undergo testing for these residues. The limits are set at 5 ng/ml for penicillin G, 10 ng/ml for amoxicillin, ampicillin, and cloxacillin, and 20 ng/ml for cefapirin (Schenck and Callery, 1998).

1.3. Tetracyclines

Tetracycline antibiotics are commonly used to treat bovine mastitis. The FDA has established levels of concern for residues of chlortetracycline, oxytetracycline, and tetracycline in milk at 30, 30, and 80 ng/mL, respectively (Olak *et al.*, 2007).

1.4. Macrolides

Macrolide antibiotics are more effective against gram-positive bacteria and are used to treat a wide range of infections. Pirlimycin hydrochloride is approved by the FDA for intramammary infusion treatment of clinical mastitis in dairy cows. A tolerance of 400 ng/mL of pirlimycin in milk has been established (Schenck and Callery, 1998).

1.5. Chloramphenicol, Florfenicol, and Thiamphenicol

These antibiotics have a broad spectrum and are suitable for treating a variety of infectious organisms. A small percentage of people exposed to chloramphenicol have been reported to develop aplastic anemia; the drug is not approved for use in food-producing animals in the United States (Schenck and Callery, 1998). Florfenicol is approved for the treatment of bovine respiratory disease in the United States. The FDA has set a level of concern for this drug in milk at 10 ng/mL (Schenck and Callery, 1998). Unlike many of the more polar antibiotics, the three antibiotics chloramphenicol, florfenicol, and thiamphenicol can be extracted from biological matrices using an organic solvent. A simple shake with ethyl acetate is sufficient to extract a small amount of chloramphenicol and florfenicol from milk (Wal *et al.*, 1980).

2. Methods for Identifying Antibiotic Residues in Milk

Due to the consequences of antibiotic residues, several laws and regulations have been enacted, including the development of methods for detecting antibiotic residues (Toldrá and Reig, 2006). Antibiotic residue detection methods are mainly divided into two groups: screening and confirmatory. Confirmatory assays are usually based on quantifying the concentration of the analyte (Cháfer-Pericás and Maquieira, 2010). Screening methods are used to determine if a sample contains antibiotic residues. If a positive result is obtained, an appropriate method is then used for confirmation, identification, and quantification. These methods detect an analyte at a concerning level and typically provide semi-quantitative results. Characteristics of these methods include lower false-positive rates, high throughput, simplicity, time-saving, low cost, and better selectivity (Liousia *et al.*, 2015). Methods commonly used for screening antibiotic residues include chromatographic, microbiological, and immunoassay methods (Yang *et al.*, 2014).

3. High-Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique that requires specialized equipment. This technique is time-consuming due to the preparation of samples and reagents and must be performed by skilled and trained personnel. Fat, protein, and sugars present in milk can compromise the correct identification of residues. HPLC is considered the gold standard method for detecting antibiotic residues due to its high sensitivity and quantitation (Vercelli *et al.*, 2023). The HPLC instrument consists of five main components: mobile phase, detector, pump, column, and sampler (manual or automatic). Samples are injected and transported through a column by the flow of the mobile phase. The pump generates the desired flow and pressure and pushes the mobile phase through the column to the detector. As a result, a signal is generated that is proportional to the amount of compounds present in the sample (Parmar *et al.*, 2021). The compound separated during HPLC can be identified, and its concentration in the sample calculated by comparing the peaks obtained from the analysis with a calibration (reference) curve

(Vercelli *et al.*, 2023). The use of HPLC for the detection of tetracycline, sulfonamides, and chloramphenicols in milk has been validated. This method was used to analyze 128 raw bovine milk samples, and oxytetracycline residues were detected in 4.9% of the samples, while amoxicillin residues were detected in 6.1% of the samples, maintaining the same performance reported in the initial phase. Kumar *et al.* (2022) performed the same method for pasteurized milk samples, but amoxicillin was not detected, possibly due to heat treatment or dilution of the samples (Kumar *et al.*, 2022).

4. Liquid Chromatography-Mass Spectrometry (LC-MS/MS)

LC-MS/MS is an advanced analytical technique that excels in identifying and quantifying antimicrobial drugs, their metabolites, or residues in food like milk. These residues are characterized with very high accuracy (Vercelli *et al.*, 2023). In this method, sample compounds are ionized, evaporated after being removed from the column, and then fragmented. They are separated based on their mass-to-charge ratio (m/z) in a mass analyzer. The detector measures the frequency or intensity of each ion with a different m/z value, which is proportional to the concentration of the analyte in the sample. Ions are typically very specific to a particular substance, allowing for precise identification and quantification. This technique is highly accurate, more so than HPLC, and is considered a high-standard and validated tool. The high accuracy of this technique enables the identification and quantification of very low levels. Additionally, it has the ability to detect multiple residues from different antibiotic classes simultaneously. The main disadvantages of this technique are its high cost, due to the purchase of equipment, long operating time, and the need to work under standardized experimental conditions (e.g., control of ionization, pH, analyte stability, etc.). Furthermore, the complexity of milk composition may lead to misleading results (Li *et al.*, 2017).

5. Ultra-High Performance Liquid Chromatography (UPLC)

It is an advanced technique that has been successfully applied for the detection of antimicrobial residues. It is used alone or in combination with electrospray ionization (ESI) and tandem mass spectrometry (UPLC-ESI-MS/MS) (Vercelli *et al.*, 2023). Recently, a study focused on the simultaneous detection of 38 veterinary antibiotic residues in raw milk using UPLC-MS/MS. The results showed that the variable recovery was 68–118% for drugs belonging to the β-lactam group, 79–118% for quinolones, 71–106% for sulfonamides, 76–116% for tetracyclines, 78–106% for macrolides, and 88–103% for lincosamides, with coefficients of variation less than 15% for day-to-day accuracy (Vercelli *et al.*, 2023).

6. Square Wave Voltammetry (SWV)

In SWV, a series of equal amplitude pulses are applied. In each forward pulse, chemicals are diffused to the electrode surface and immediately reduced or oxidized. During the reverse pulse, the recently oxidized or reduced chemical species return to their initial state in a reversible reaction. If the system is irreversible, no reaction occurs. Therefore, the current values just before and at the end of each pulse are measured, and the net or resultant currents are plotted as a function of the corresponding potential in a staircase waveform, resulting in a Gaussian signal. The resulting signals are larger than the forward and reverse signals due to the significant reduction of capacitive currents. This provides high sensitivity and better selectivity than other electroanalytical techniques. Additionally, this technique offers the advantages of rapid analysis, good analytical frequency, low cost, and ease of operation (Megale and Souza, 2023).

Due to the presence of functional groups such as amide, amine, aromatic rings, carbonyl, double bonds, triple bonds, and sulfur bonds in antibiotics and other pharmaceutical compounds, they can be identified using the SWV technique. These compounds can be oxidized or reduced, providing a measurable electrical signal at a specific potential. This allows for both qualitative and quantitative analysis. Quinolones and fluoroquinolones contain a central amine group in their structure with a non-bonding electron that acts as a donor and can be oxidized electrochemically. The values obtained from this process are directly proportional to the antibiotic concentration. The oxidation reaction occurs at the secondary amine group in these antibiotics, leading to the formation of hydroxylamine. Cephalosporins, a class of β-lactam antibiotics, are chemically and electrochemically active due to their β-lactam ring structure. They can easily undergo hydrolysis in the environment or in an electrochemical cell, with peak currents reflecting the concentration of cephalosporins. Similarly, penicillins also contain a β-lactam ring that can undergo irreversible electron transfer through electrochemical oxidation. Peak currents and potentials can be utilized for the quantification and identification of penicillin antibiotics in food samples (Zhu *et al.*, 2023; Baezzat *et al.*, 2021).

7. Microbiological tests

In this method, there is no need for sample preparation. When an inhibitory zone appears in the reference bacterial cultures, the milk sample is considered positive. The diameter of the inhibitory zone must

be measured to interpret the results (Vercelli *et al.*, 2023). This technique has low specificity, which may lead to false results. High somatic cells or pH changes in the case of mastitis may result in false negative results. Additionally, the amount of antibiotic residues cannot be quantified with this technique. Moreover, only a small number of antibiotic families can be identified using this method. However, this method is still the focus of numerous studies aimed at improving the current method due to its short execution time, versatility with different animal origin products (such as eggs, honey, raw, pasteurized, and bulk milk), and low costs (Vercelli *et al.*, 2023). The development of this technique has enabled the design of specific bacteria, such as Geobacillus stearothermophilus, Bacillus megaterium, and Bacillus licheniformis, for the detection of beta-lactams, macrolides, tetracyclines, quinolones, and sulfonamides, respectively. These bacteria can detect these substances at levels close to the maximum residue limits (MRL) (Vercelli *et al.*, 2023). In this technique, all the elements are placed in a test tube and milk is added to it. The tube is then incubated at the appropriate temperature to provide conditions for bacterial growth. In the absence of antibiotic residues, changes in turbidity and color due to the acidification of the pH of the medium, which indicate the normal growth of bacteria, can be observed visually. For example, there is a change in color of the medium from yellow to black for Geobacillus stearothermophilus and from pink to blue for Bacillus licheniformis. In the presence of antibiotic residues in the milk, bacterial growth is inhibited and no changes are visible. A similar method based on the growth of Bacillus subtilis is able to detect the presence of sulfonamides (Nagel *et al.*, 2013). Several kits based on this technique are now commercially available. Two of the most popular are: (a) the Dulcotest ST-NP, which can detect beta-lactams, aminoglycosides, macrolides, sulfonamides, tetracyclines, and diaminopyrimidines, and (b) the Charm® QUAD1 test, which only detects beta-lactams, quinolones, sulfonamides, and tetracyclines. Both tests are capable of detecting residues at the minimum MRLs and report a sensitivity of 95% (Vercelli *et al.*, 2023).

7.1. Optical Biosensors

Optical biosensors are a prominent and widely used type of sensor, prepared by integrating a biological detection element with an optical transducer. These devices operate based on optical transmission, collecting signals from one or more analytes to quantify crucial details in optical radiation, including frequency, amplitude, and polarization (Dezhakam *et al.*, 2024). The advantages of these biosensors include remarkable sensitivity and accuracy, the ability to detect a specific analyte, low background noise, satisfactory limit of detection (LOD), and rapid analysis. These biosensors are more cost-effective due to the support of multiple different biosensors with a fixed hardware system, along with a complementary metal oxide semiconductor (CMOS) camera. Additionally, optical biosensors are easy to transport and allow for remote detection to prevent contamination of the device by the analyte. This feature makes them a viable option for observing biological samples (Dezhakam *et al.*, 2024). Using optical biosensors and sensors, a variety of analytes such as proteins, DNA/RNA derivatives, bacteria, viruses, tissues, and drugs can be detected at low concentrations. Similar to electrochemical biosensors, nanoparticles such as AuNPs can be utilized in these biosensors to amplify the sensitivity of optical signals. Optical biosensors can be classified into label-free and label-based detection methods (Dezhakam *et al.*, 2024). In the label-free system, signals are generated as a result of the direct interaction between the transducer and the sensor analyte, without the presence of any conjugated label. In the label-based method, special materials such as fluorescent, colorimetric, and isotopic labels are used to obtain optical parameters. This method is more expensive and specialized compared to the label-free options (Khansili *et al.*, 2018).

Various optical biosensing techniques have been characterized to date, including fluorescence, colorimetry, spectrofluorometry, surface plasmon resonance (SPR), and Surface-Enhanced Raman Spectroscopy (SERS). Altintas utilized a combination of nanomolecularly imprinted polymer and surface plasmon resonance (SPR) techniques in his sensor structure for the detection of glycopeptide antibiotics, such as vancomycin, in milk samples (Dezhakam *et al.*, 2024).

The SPR technique is inspired by the phenomenon of surface plasmon. Plasmon refers to the oscillations of electrons in a metallic conductor when high-energy electrons pass through it. In this technique, polarized light is incident at a certain angle on the surface of a conductive material such as metal at the interface of two media (generally glass and liquid). This interaction between free electron density fluctuations and electromagnetic waves between the surface of the dielectric film and the metal leads to the generation of surface plasmon. By measuring the change in angle, reflection, or wavelength over time, various details about the concentration, equilibrium, and movement of molecular interactions can be obtained (Dezhakam *et al.*, 2024).

8. Immunological Methods

8.1. ELISA

The enzyme-linked immunosorbent assay (ELISA) technique was first described by Engvall, Johnson, and Perlman in 1971. It is based on antigen-antibody binding, which produces a colorimetric reaction due

to the presence of a chromophore bound to the antigen. The ELISA technique can be designed as qualitative (positive/negative result) or quantitative (result as a measurement or concentration). There is also a semi-quantitative test that provides different levels of positivity and negativity that must be compared with a reference scale. Several commercially available ELISA-based kits are used for the rapid detection of various families of antibiotics in bovine milk. These kits can be used as a screening test by veterinarians and dairy industry personnel to examine the milk of one or more cows due to their ease of implementation and low cost (Dezhakam *et al.*, 2024). If the ELISA test result is positive (meaning residues above the MRL are detected), it is necessary to perform a confirmatory test using more specific and sensitive tests such as high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS/MS). The ELISA technique has recently been validated for the detection of β-lactams in milk (Vercelli *et al.*, 2023).

A new technique based on magnetic immunoreactivity, inspired by antigen-antibody interaction, has been developed for the detection of kanamycin and penicillin in dairy milk. This technique shows very low limit of detection (LOD) and high sensitivity. Pretreatment and dilution steps of milk samples are necessary for this technique. However, it is important to note that this technique is still in the proof of concept stage and has not yet been commercialized (Schenck and Callery, 1998). An ELISA-based microarray assay has recently been approved under specific European regulations to simultaneously detect norfloxacin, tetracycline, lincomycin, and streptomycin in milk samples, achieving very high accuracy (ranging from 77.6% to 116.4% for different antibiotics). Its disadvantage is that it requires specialized and trained personnel (Vercelli *et al.*, 2023). So far, this method has been proposed for control programs in the dairy industry (Du *et al.*, 2019).

8.2. Lateral Flow Immunoassay (LFIA)

This technique, due to its simpler sample preparation, requires less time compared to ELISA and allows for the analysis of large amounts of samples with a low investment cost, providing rapid results. In recent years, this technique has been widely used for the detection of antibiotics in milk, such as beta-lactams, tetracyclines, streptomycin, and chloramphenicol (Vercelli *et al.*, 2023).

8.3. Radioimmunoassay (RIA)

Radioimmunoassays have been widely used in the past for detecting antibiotic residues in food because of their rapid performance and low limit of detection (LOD). However, the use of this technique has been limited to only a few clinical purposes due to the short half-life of the radioisotopes used to label the analyte and the potential hazards of using radioisotopes for personnel and the environment (Vercelli *et al.*, 2023).

8.4. Fluorescence Immunoassay

This method is based on the binding of a fluorophore to the antigen, and its detection is accurate and reliable. However, background signal can interfere with the emission, potentially leading to ambiguous results (Schenck and Callery, 1998). This method has been widely used for the detection of fluoroquinolones, beta-lactams, and tetracyclines in milk (Vercelli *et al.*, 2023).

9. Conclusion

Given the adverse effects of antibiotic residues on health and the development of antibiotic resistance, it is necessary to detect these residues in food using effective methods. The methods mentioned in this article are desirable for detecting antibiotic residues in milk. It is hoped that in the near future, new and more effective methods for detecting antibiotic residues in food and other areas will be presented.

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