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Detection of Antibiotic Residues of Tetracycline, Oxytetracycline and Chlortetracycline in Animals' Raw Milk in Hamedan Province, Iran in 2022 Using the Four-Plate Test, ELISA, and HPLC Techniques

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ABSTRACT

Antibiotic resistance has become a health concern as it is associated with the death of numerous people worldwide. Milk safety is one factor that guarantees the quality of dairy products. This study was designed to determine Tetracycline (TC), Oxytetracycline (OTC), and Chlortetracycline (CTC) residues in raw milk from animals in Hamedan, Iran using a four-plate test (FPT), enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC) techniques. Over a two –year Period a cross-sectional sampling was conducted, during which 246 unprocessed raw milk samples were collected from dairy farms and milk collection centers in different regions of Hamedan, the western part of Iran. FPT was used as the initial screening tool for the presence of antibiotics. Positive samples were subsequentlyanalyzed for antibiotic residue using ELISA. Finally, the HPLC method was applied to identify the type and quantity of Tetracycline residues. In the primary evaluation, forty-seven samples (19.11 %) tested positive for antimicrobial residues using FPT. ELISA analysis indicated that 29.79 % (14/47) of these samples contained TCs residues higher than the maximum residue limit (MRL) suggested by EU (100 µg/L). The average TCs residue in positive samples was 98.43±6.86 µg/Land the lowest and highest levels were 100.59 µg/L and 129.56 µg/L, respectively. Having used HPLC, the overall average of TCs level was calculated as 105.73±7.25 µg/L (TC=100.67, OTC=103.38, and CTC=107.11 µg/L). The detection of antibiotic residues in animal products highlights the need for monitoring these residues in milk and other animal-derived food products. Training farmers on the correct use of drugs, especially antibiotics, is recommended. Additionally, acomprehensive protocol for regularly evaluating livestock products is necessary to prevent high-contamination products from entering the production cycle.

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1. Introduction

Antibiotic resistance has become a health concern as it is associated with numerous deaths worldwide (1). Population growth results in a greater need for food. Dairy products rank among the most essential foods for meeting a person's nutritional needs. Dairy products come from a variety of sources, both modern and traditional, including domestic animal milk. The health of this product is critical because unprocessed raw milk is the primary ingredient in other dairy products (2).

The presence of residuessuch as antibiotics, hormones, pesticides, disinfectants, insecticides, mycotoxins, and heavy metals can endanger human health (3). Despite regulatory restrictions, antibiotics are still used as food preservatives to stimulate growth and boost productivity in animals and poultry. In addition to their veterinary care use today, continuous exposure of humans to antibiotic residues is associated with the adverse effects such as allergic reactions, disturbing normal intestinal flora, and transferring antibiotics resistance genes (ARGs) or antibiotic-resistant bacteria (ARB) from animals to humans (4, 5).

Consumers' health is significantly threatened in many of developing nations since maximum residue limit (MRL) established byEuropean Union (EU) and World Health Organization (WHO) have not been clearly defined (6). Due to increasing bacterial resistance, medication residues can be present in animal-derived foods when prescribed dosage of pharmaceutical compounds is not followed and withdrawal periods for using natural and synthetic antimicrobials are not observed (7). Besides health concerns, antibiotic residues in milk may also cause industrial problems during the manufacturing of milk products, especially in fermented goods (8).

Tetracycline (TTR), chlortetracycline (CTC), and oxytetracycline (OXY) are commonly used drugs in veterinary medicine. They are widespread antibiotics that are effective against a broad range of infectious diseases in animals (9). The EU's recommended MRL for Tetracycline compounds (TCs) residues in raw milk is 100 µg/L (6). Residual levels of TCs exceeding this limit pose significant risks to Patients, fetuses, newborns, and children under the age of 12. Therefore, it is crucial to monitor and identify TC antibiotic residues in milk (5). There are different methods for detecting the antibiotic residue in milk. The most common include microbiological, immunochemical methods such as Enzyme-Linked Immunosorbent Assay (ELISA), Chemiluminescence Immunoassay (CLIA), Radioimmunoassay (RIA), Colloidal Gold Immunoassay (CGIA) and Fluorescence Immunoassay (FIA), as well as physicochemical methods such as fluorescence spectrophotometry (FS) and high-performance liquid chromatography (HPLC) (7-10).

Microbiology methods are among of the most cost-effective, time-efficient, and high-sensitivity methods for rapid detection of antibiotic residues in food. Subsequent evaluations can be performedusing immunochemical and chromatographic techniques. Additionally, the four-plate test (FPT) method is useful for the qualitative detection of antibiotics or a group of antibiotics at levels higher than the MRL. However, this method is time-consuming (18 hours) and not suitable as a rapid detection test. In the FPT, the milk samples are applied to four agar media plates inoculated with *Bacillus subtilis* spores (at pH 6, 7.2, and 8) and *Micrococcus luteus* (at pH 8). Inhibition zones of one or both microorganisms indicate the diffusion of an antibiotic agent (11, 12). Over the past two decades, ELISA has been developed and used for semi-quantitative detection of antibiotic residues such as TCs in dairy products (13). Bioassay techniques are less precise than others due to the long evaluation period and the lack of accurate diagnosis of the type of antibiotic. Tetracyclines have been detected and measured quantitatively and accurately in milk and other animal tissues by using chromatographic methods such as thin-layer chromatography (TLC), capillary electrophoresis (CE) and HPLC (7, 14, 15).

Due to the increasing trend of the animal husbandry industry and the subsequent use of veterinary drugs to control and treat various diseases, the main objective of this study was to investigate the presence of TCs residues in cow milk in Hamedan, Iran, using FPT, ELISA, and HPLC Techniques.

2. Materials and Methods

2.1. Location of Study

Hamedan province (34.77° N and 48.58° E),covering an area of 19,546 km^2, is located in the western part of Iran. Animal husbandry and agriculture are the main occupations of people in this region. A total of 70 dairy farms ,with an estimated cattle population of 30,000, are distributed across Hamedan province. Additionally, sheep

and goats are about two million heads. The daily milk production in this region is 600 tons (Figure 1).

2.2. Sampling

In this descriptive cross-sectional study, 246 raw milk samples were randomly collected from farms and milk collection centres in 2022. Twenty mL of milk were collected at each location in sterile laminated polyethene sampling containers and was quickly transported to the laboratory of Hamadan University of Medical Sciences for the evaluation of antimicrobial residues. All sample characteristics were recorded during the sampling time.

2.3. Sample Preparation

Initially, the samples were placed on a shaker to reach laboratory temperature and homogenized. The samples were tested according to hygiene considerations.

2.4. Detection of Inhibitory Substances

2.4.1. Microbiological Assay

The FPT method was used to examine the presence of tetracycline, oxytetracycline, and chlortetracycline. *Bacillus subtilis* PCTT 1204 and *Micrococcus luteus* PCTT 1408 were procured from the Iranian Research Organization for Science and Technology (IROST) and were used in the FPT. These bacteria were cultured in a Nutrient Agar medium (Ibresco, Iran). After bacterial proliferation, a suspension was prepared in a Nutrient Broth medium (Ibresco, Iran) at a 0.5 McFarland test concentration. To prepare the culture medium, according to the manufacturer's instructions, 38 g of dry Mueller Hinton Agar (MHA)(Merck, Darmstadt, Germany) powder was mixed with 1L of distilled water and heated with a magnetic stirrer until the boiling temperature was reached.

Then, the pH was adjusted to 6, 7.5, and 8 using a digital pH meter with acetic acid and sodium hydroxide. The resulting medium was sterilized in an autoclave at 121°C for 15 minutes. To increase the method's sensitivity and detect sulphadimidine residues, trimethoprim (ASICO, Iran) was added to the pH 7.2 MHA to a final concentration of 0.05 mg/L. 25 mL of MHA were poured into each sterile petri dish (diameter, 90 mm). 50 µL of *B. subtilis* were spread onto fresh MHA plates at three pH levels of 6, 7.2, and 8, and 50 µL of *M. luteus* at pH = 8 (0.5 McFarland)were also applied In the next step, we placed a blank disc paper on the surface of bacteria cultures in MHA. Afterwards, 25 µL of milk samples were separately loaded into the discs. Paper discs (6 mm diameter; Padtantab, Iran) containing different antibiotics were placed in the center of each Petri dish. The media seeded with *B. subtilis* were incubated overnight at 30°C,while the medium seeded with *M. luteus* was incubated at 37 °C for 18-20 hours. After incubation, plates were examined for inhibition zones around the milk discs. Inhibition zones of 2 mm or more in width were recorded as positive. All experiments were performed in triplicate (11, 16).

2.4.2. Detection of Tetracyclines with ELISA

All samples showing inhibition zones wider than 2 mm on at least one plate with *B. subtilis* were further examined for TCs using a commercially available ELISA kit (Ridascreen Tetracycline ELISA kit, r-biopharm, Germany). Milk samples were centrifuged at 3000 g for 10 minutes. The cream on top of the milk was separated by a Pasteur pipette. The remaining milk was diluted 1:10 in a new microtube containing sample buffer 2, included in the kit.

50 µL of concentrated tetracycline standard were diluted with 450 µL of dilution buffer. 50 µL of the standard and the prepared sample were added to the well in pairs to obtain the number of two biological replicates. 50 µL of anti-tetracycline-antibody were added to each well,which were then incubated for one hour at 20-25°C. The liquid was removed from each well and washed with 250 µL of wash buffer (PBS-Tween buffer). This process was repeated three times. 100 µL of conjugate were added to each well and gently mixed by manual shaking and then incubated at 20-25°C for 15 min. The wells were washed three times with a wash buffer. 100 µL of substrate/chromogen were added to each well, mixed thoroughly by manual shaking, and incubated at 20-25°C for 15 min. 100 µL of stop solution were added to each well and mixed thoroughly by manual shaking, and the absorbance was read at 450 nm (Microplate reader, Stat Fax 4300, USA). OD values were recorded, and the percentage of absorbance was calculated as follows: % absorbance= absorbance standard per sample/absorbance zero standard × 100. The calibration curve was plotted between the standard concentration and OD.

2.4.3. HPLC Analysis

2.4.3.1. Preparation of Standard Curves

To confirm the exact concentration of TCs contamination in positive ELISA samples, the HPLC analysis was performed. The optimized method was validated according to the European Commission Directive 2002/657/EC (6). Stock solutions (100 µg/mL) of TCs and working standards (50-400 ng/mL) were prepared as a mixture of methanol, acetonitrile, and 50

Figure 1. Location of Hamedan province, sampling area, in the western part of Iran.

mM oxalic acid (10: 20: 70 %). Mixed standard solutions were then prepared for simultaneous calibration and calculation of TCs residues (9).

2.4.3.2. Sample Preparation for HPLC

Citrate-phosphate buffer (pH 4.1) was prepared by adding 11.8 g of the citric acid monohydrate and 13.72 g of disodium hydrogen phosphate dehydrate to 33.62 g of ethylene-diaminetetraacetic acid disodium salt (EDTA 0.01 M) in a final volume of one litre. To 5 ml of homogenized milk sample, Two mL of 20 % TCA (Trichloroacetic acid-Merck- Germany) and 20 mL of Citrate-phosphate buffer were added. The mixture was thoroughly agitated and centrifuged at 4000 rpm for 20 minutes. The floating lipid layer was removed, and the resultant supernatant was applied to the solid-phase extraction cartridge (SPE HLB C18). The SPE cartridge was first activated with 3 ml methanol at a flow rate of 3 ml/min , then rinsed with 2 ml of double-distilled water. The centrifuged sample solution was loaded onto the SPE cartridge at a flow rate of 5 ml/min. After loading each sample, the cartridge was treated with 2 ml of 5 % methanol in double-distilled water., The analytes were performed with 2 ml of HPLC -grade methanol at a rate of 4 ml/min, and the residue was dissolved in 1 ml of mobile phase (15, 17).

2.4.3.3. HPLC condition

The UHPLC-KUNAER system (model A69420, Germany) was equipped with a UV-vis detector (model 2500, Germany). Separation of milk samples was carried out under isocratic conditions using a C18 Column(250 mm× 4.6 mm I.D., containing 5 µm particles) and a binary pump. The mobile phase consisted of methanol, acetonitrile, and 50mM oxalic acid (10: 20: 70 % V/V), and was filtered through a 0.45-µm micro-filter, at an adjusted flow rate of 1 ml/min, and wavelength of 353 nm with an injection volume of 20 µL. TC concentration was calculated by measuring the areas under the peaks rather than the relevant peaks generated by the standard TCs (17).

2.4.3.4. Validity Parameters

Validity parameters ,including recovery, linearity, the limit of detection (LOD), and the limit of qualification (LOQ), were obtained. Calibration curves of mixed standard TCs were provided for five concentration levels (50, 100, 200, 300, and 400 µg /L) in a blank milk sample with three replicates. The LOD and the LOQ were determined based on the ratio of S/N=3 and S/N =10, respectively. A recovery test was performed using the spiked blank milk in three concentration levels (100, 200, 300 µg /L) of the standard mix (18).

3. Results

3.1. FPT

In the primary evaluation of the samples, 19.11 % (47/246) sholwed positive reaction to the antibiotic residues. Table 1 presents the numbers of positive results for plates seeded with *B. subtilis* and *M. luteus*. Among the 47 samples positive for antibiotic residues, 13 milk samples (27.66 %) showed inhibition zone ≥ 2 mm on all 3 plates (pH 6, 7.2, and 8). Ten samples (21.28 %) were positive only on one of 3 plates (pH 6) ,and 14 samples (29.79 %) were positive only on two plates (pH 6 and 7.2).

3.2. ELISA

After initial screening using the FPT, a semi-quantitative ELISA method was performed to determine

Table 1. A number of samples containing substances inhibitory to *B. subtilis* and *M. luteus*.

Microbial species	pH	Inhibition zone (mm)		
		1>	1-2	2<
	6	199	26	21
	7.2	200	26	20
	8	212	25	9
B. subtilis	6+7.2	201	25	20
	6+8	224	13	9
	7.2+8	223	14	9
	6+7.2+8	224	13	9
M. luteus	8	212	16	18

the residue level of antibiotics in the positive milk samples. The resultsindicated that 29.79 % (14/47) of samples contained a TC level higher than that of MRLsuggested by EU (100 µg/L). The average TCs residue in positive samples was 98.43±6.86 µg/L. Additionally, the lowest and highest recorded levels were 100.59 µg/L and 129.56 µg/L, respectively.

3.3. HPLC-UV

The method was validated according to the criteria specified in EU Commission Decision 2002/675/EC (Decision 2002). The validity parameters, including LOD, LOQ, Regression coefficient, and Retention Time (minutes) are listed in Table 2. Also, the obtained recovery percentages are presented in Table 3. The highest and lowest recovery rates were obtained 81.45 % and 64.33 % for tetracycline and chlortetracycline, respectively. The screening test confirmed that 29.79 % of the samples were positive for TCs residues at levels above the established MRL.

In the HPLC analysis, all 14 positive samples in ELISA were contaminated with TCs. Among the TCs, CTC was the most frequently detected, as it was found to be present in all tested samples (n=14) and covered 100 % of the total concentration of measured TCs residues. The average residue of CTC, TC, and OTC were 107.11, 100.67, and 103.38 µg/L, respectively (Table 4). The overall average concentration of the three analyzed antibiotics among the positive samples (105.73 µg/L) was just slightly higher than the allowed limit, taking into account that the standard limit of TCs residues in milk specified by MRL-EU is 100 µg/L (6).

4. Discussion

A significant proportion of antibiotics produced worldwide are used in animals to control diseases. In Iran, the consumption of antibiotics, especially tetracyclines, in animal husbandry is notable compared to other countries (1). According to the Food and Agriculture Organization (FAO) of the United Nations, milk is one of the most widely consumed foods that provides a large part of the daily needs of humans, especially for children (6). However, the adverse effects associated with the use of veterinary drugs, as well as antibiotic resistance, are considered to be a major threat to human health. Therefore, food source must be free from any contamination (2). In drug residues detection, microbial test can be used as a primary screen to confirm the presence of a wide range of substances that inhibit the growth of microorganisms. The results of microbial assays can contribute to identifying the specific a positive role in confirming the type of antibiotic present. The antibacterial properties of antibiotics or their metabolites in animals vary depending on the type of compound used, as well as drug administration; so immunochemical methods can be used for alternative screening purposes (19).

The immunochemical tests cannot be considered as a final tool for determining the antibiotics as the technique is highly sensitive and may result in a significant number of false positives. These tests can also help determine the specific type of antibiotic group detected by microbial tests (3). The observation of inhibition zone around samples in the FPT is only possible when the level of antibiotic residues exceedes the allowed limit. The sensitivity of FPT is high but cannot detect residues below or within the permissible limits recommended by the EU. Changing the pH of the culture medium and the type of bacteria used have the greatest impact on revealing the inhibitory effects of antibiotics in FPT (16).

Table 2. Validity parameters in analytical determination of tetracyclines residues in milk samples.

Parameter	Oxytetracycline	Tetracycline	Chlortetracycline
LOD µg/L	1.52	1.29	1.95
LOQ µg/L	4	4	5
Regression coefficient	0.995	0.998	0.994
Retention time (min)	4.23	4.91	7.49

Table 3. Recovery (%) of tetracycline spiked in different concentration levels in milk samples.

	different concentration levels of the spiked sample (µg/L)			
	100	**200**	**300**	**Mean**
Oxytetracycline	65.43±7.2	59.96±6.2	70.09±1.9	65.16±5.1
Tetracycline	87.72±3.1	85.51±1.9	71.12±9.12	81.45±4.71
Chlortetracycline	67.2±2.8	60.94±3.1	64.85±2.9	64.33±2.93

Table 4. Tetracycline residues in milk samples (µg/L).

	Oxytetracycline	Tetracycline	Chlortetracycline	Total residues in positive samples
Number of sample	7 (50%)	3 (21.43%)	14 (100%)	14 (29.79%)
Mean	103.38±1.07	100.67±0.11	107.11±7.65	105.73±7.25

In this study, three methods, namely, FPT, ELISA, and HPLC, were investigated in tracking and determining the antibiotic residues in raw milk samples. Regarding the FPT, 19.11 % of samples were positive. On the plates seeded with *B. subtilis* at pH 6, 7.2, and 8,and on plate seeded (pH 8) with *M. luteus* at pH 8, 27.66% and 53.19% of milk samples, respectively, showed inhibition zone ≥ 2, indicating the presence of antibiotic residues.

In a study from Tehran, the capital of Iran, 8.7 % of pasteurized milk samples were positive for tetracycline and oxytetracycline residues (20). In the study by Moghaddam et al (21) from Northeast Iran, 28.7 % of milk samples were found to contain antibiotic residues.

In another study from Zanjan, Iran, according to MRLs, antibiotic residues in milk samples were present in 31.25 % and 9.38 % of the industrial and rural products, respectively. Sulfonamide, beta-lactam, and tetracycline were the most commonly detected antibiotic residues (22).

Regarding Nematiniko et al's (13) study from Qazvin province, Iran, using Copan and ELISA kits, 48.91 % of milk samples were positive for antibiotic residues. In a meta-analysis by Bahramian et al (1) from Iran, antibiotic residues were detected in 26 % of raw milk samples and 21 % of pasteurized milk samples. Furthermore, the prevalence rates reported using screening, ELISA, and HPLC techniques were 28%, 43%, and 27%, respectively (5). A study from Brazil (22) reported that (23) among the positive samples, 6 % were contained both tetracycline and β-lactam antibiotics, while 11 % were positive only for beta-lactams. The consumption of milk containing antibiotics plays an important role in the development of drug resistance. Proper withdrawal times for of antibiotics can prevent the release of antibiotic residues in milk from livestock. Educating farmers about the risks of overusing drugs and antibiotics in livestock products is crucial for improving the current situation (3).

As previously noted, the pH of the culture medium significantly influences the results.

The highest diameter of the inhibition zone was observed at the lowest pH tested indicating the presence of tetracyclines, although beta-lactam antibiotics also behave in the same way. Most beta-lactam antibiotics, especially penicillin, are less stable in storage conditions (2, 3). Additionally, since tetracyclines were detected in the majority of plates seeded with *B. subtilis*, the remaining semi-quantitative antibiotics were quantified

using a commercial ELISA kit capable for detecting various tetracyclines. As previously noted, the pH of the culture medium significantly influences the results. FPT cannot identify antibiotic residues below the MRL due to the dispersion of compounds within the culture medium, which affects the sample matrix. In the ELISA evaluation, 29.79 % of positive samples in FPT showed antibiotic residues exceeding the maximum residue limit (MRL) set by the European Union for milk (100 µg/kg). The average tetracycline residue in these samples was 98.43±6.86 µg/L. In an ELISA investigation on milk from Lebanon, the maximum standard concentrations of tetracycline and penicillin were 1.80 ng/kg and 4.00 ng/kg, respectively (23). In a comprehensive investigation of milk residues, the β-lactam group was the most frequently detected (36.5 %), followed by tetracyclines (14 %), fluoroquinolones (13.5 %), sulfonamides (12.6 %), and aminoglycosides (10.4 %) (8).

In this investigation, HPLC is used to detect tetracycline residues in 29.79 % of the positive samples during the ELISA stage. Tetracyclines were detected in the samples with an average concentration of 105.73±7.25 µg/kg. In the Cinquina et al. study (17), tetracycline levels were calculated to be 81.1 % in cow's milk using the HPLC/PDAD technique. In another report, 19.8 % of the pasteurized and sterilized milk samples had antibiotic residual levels exceeding EU MRLs' maximum residue limits. Conversely, 14.97 % of samples were below the EU MRLs standards. Additionally, no significant differences were observed in the detection level of antibiotic residual and sampling seasons, as well as between sterilized and pasteurized samples (2). Milk contamination poses risks to both dairy products and human health (24).

The differences in tetracycline residues between the present study and other reports may be due to various of factors, including differences in sample size, laboratory diagnostic methods, drugusage practices, withdrawal times, monitoring approaches, and health protocols in each area. The presence of pharmaceutical residues in dairy products is unacceptable due to the potential harm to consumers and the negative impact on the food industry. Based onour findings, a significant proportion of milk samples were positive for antibiotic residues. For this reason, to lessen the risk of contamination in the Hamedan region, Iran, specific precautions and comprehensive monitoring procedures are required. Currently there is no appropriate program or essential regulation on milk processing plants and milk collecting centers for evaluating antibiotic residues during milk collection and delivery. It is recommended to train farmers on proper drug use, especially antibiotics, and to ensure adherence to withdrawal period from milk according to the instructions of the farm veterinarians. Furthermore, development a comprehensive protocol for periodic and regular evaluations of livestock products in terms of residues and preventing products with high contamination from entering the production cycle seems crucial. Further research is necessary to identify potential programs aimed at reducing and controlling the concentration of contaminants in milk.

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Authors' Contribution

Study concept and design: M.A.
Data acquisition: F.M and M.V
Data analysis and interpretation: M.K and J.G
Drafting of the manuscript: M.A and J.G
Critical revision of the manuscript for important intellectual content: M.A, F.M, and J.G.
Statistical analysis: M.K
Administrative, technical, and material support: M.A, F.M and J.G
All authors read and approved the final manuscript.

Ethics

Not applicable.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Data Availability

The data supporting this study's findings are available upon request from the corresponding author.

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