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

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 of animals from Hamedan, Iran using a Four-plate test (FPT), enzyme-linked immunosorbent assay (ELISA), and high-performance liquid chromatography (HPLC) techniques. Cross-sectionally over two years, 246 unprocessed raw milk samples were taken from dairy farms and milk collection centres of different regions of Hamedan, the western part of Iran. FPT was the first tool for screening the presence of antibiotics. Then, the positive samples were analyzed for antibiotic residue using ELISA. Finally, the HPLC method was applied to determine the type and amount of Tetracycline residues. In the primary evaluation, forty-seven (19.11 %) samples were positive for antimicrobial residues using FPT. ELISA analysis indicated that 29.79 % (14/47) of samples had a level of TCs higher than the maximum residue limit (MRL) suggested by EU (100 µg/L). The average TCs residue in positive samples was calculated 98.43±6.86 µg/L. The lowest and highest levels were 100.59 µg/L and 129.56 µg/L, respectively. Finally, the average TCs was calculated 105.73±7.25 µg/L (TC=100.67, OTC=103.38, and CTC=107.11 µg/L) using HPLC. The detection of antibiotic residues in animal products highlights the need for monitoring such residues in milk and other animal-origin food products. Training farmers for the correct use of drugs, especially antibiotics, is recommended. A comprehensive protocol for regularly evaluating livestock products is necessary to prevent high-contamination products from entering the production cycle.

Keywords: Milk, Antibiotic residue, Tetracycline, Iran

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 residues, such as antibiotics, hormones, pesticides, disinfectants, insecticides, mycotoxins, and heavy metals can endanger human health (3). Antibiotics are still used as food preservatives to stimulate growth and boost the productivity of animals and poultry. This is in addition to their use in veterinary care today, despite regulatory authorities' restrictions on the practice of prescription antibiotics (2). As mentioned in many studies, the continuous exposure of humans to antibiotic residues is associated with the eventuality of expanding 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 jeopardized in the majority of developing nations since the maximum residue limit (MRL) that the European Union (EU) and World Health Organization (WHO) allow has not been established (6). Due to increasing bacterial resistance, medication residues can be found in animal-based foods if the required dosage of prescribed pharmaceutical compounds is not followed and the withdrawal periods for using natural and synthetic antimicrobials is not observed (7). In addition to all the health concerns stated, it is also possible that antibiotic residues in milk might cause industrial problems in the manufacturing of milk products, especially 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 recommended MRL by the EU for Tetracycline compounds (TCs) residues in raw milk is reported 100 µg/L (6). Patients, fetuses, newborns, and children under the age of 12 are at significant risk if the residual level of TCs in dairy products exceeds the permitted limit. 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 ones are microbiological, immunochemical such as enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CLIA), radioimmunoassay (RIA), colloidal gold immunoassay (CGIA), and fluorescence immunoassay (FIA) physicochemical methods such as fluorescence spectrophotometry (FS) and high-performance liquid chromatography (HPLC) (7,10).

Microbiology methods are one of the most cost-effective, time-efficient, and high-sensitivity methods in the rapid detection of antibiotic residues in food materials. In the next step, the

samples can be evaluated using immunochemical and chromatographic techniques. In addition, the four-plate test (FPT) method is useful for the qualitative detection of an antibiotic or a group of antibiotics whose level is higher than the MRL. However, this method is time-consuming (18 hours) and therefore not suitable as a rapid detection test. 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 depict the diffusion of an antibiotic agent (11,12). Over the past two decades, ELISA has been developed and used for the 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 growing trend of the animal husbandry industry and the subsequent use of veterinary drugs to control and treat various diseases, the main objective of the 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; an area of 19,546 km^2) is located in the west 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 in Hamedan province; additionally, sheep and goats are about two million heads. The milk production is 600 tons daily (Fig. 1).

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

2.2. Sampling

In this descriptive cross-sectional study, 246 raw milk samples were obtained randomly from farms and milk collection centres in 2022. Twenty mL of milk was collected in each location in the sterile laminated polyethene sampling containers and was quickly transferred to the laboratory of Hamadan University of Medical Sciences for evaluation of antimicrobial residues. All of the 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 homogenised. The samples were tested according to hygiene considerations.

2.4. Detection of Inhibitory Substances

2.4.1. Microbiological assay

The FPT method was adopted to examine the presence of tetracycline, oxytetracycline, and chlortetracycline. *Bacillus subtilis* PCTT 1204 and *Micrococcus luteus* PCTT 1408 were purchased 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) of 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 with

1L of distilled water was mixed 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 obtained compound was sterilized in an autoclave at 121 °C for 15 min. Trimethoprim (ASICO, Iran) was added to the pH 7.2 MHA to a final concentration of 0.05 mg/L to increase the method's sensitivity and detect the sulphadimidine residues. 25 mL of MHA was poured into each sterile petri dish (diameter, 90 mm). 50 µL of *B. subtilis* was spread onto fresh MHA plates at three pH levels of 6, 7.2, and 8, and also 50 µL of *M. luteus* at pH = 8 (0.5 McFarland). 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 centre of the Petri dish. Media seeded with *B. subtilis* were incubated overnight at 30 °C. The medium seeded with *M. luteus* was incubated at 37 °C for 18-20 h. After incubation, plates were inspected 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 examined further for TCs with a commercially available ELISA kit (Ridascreen Tetracycline ELISA kit, r-biopharm, Germany). Milk samples were centrifuged at 3000 g for 10 min. The cream on top of the milk was separated by a Pasteur pipette. The remaining milk was diluted 1:10 in a new microtube with sample buffer 2, included in the kit. 50 µL of concentrated tetracycline standard was 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 was added to each well and 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 was 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 was added to each well, mixed thoroughly by manual shaking, and incubated at 20-25 °C for 15 min. 100 µL of stop solution was 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 obtained, and the percentage of absorbance was calculated as follows: % absorbance= absorbance standard per sample/absorbance zero standard × 100. The calibration curve between the standard concentration and OD was then drawn.

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 method 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 mM oxalic acid (10: 20: 70 %). Then mixed standard solutions were prepared for the 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. Two mL of 20 % TCA (Trichloroacetic acid-Merck- Germany) and 20 mL of Citrate-phosphate buffer were added to 5 ml of homogenized milk sample. The mixture was agitated thoroughly and centrifuged at 4000 rpm for 20 min. The floating lipid layer was removed and the resultant supernatant was applied to the solid-phase extraction (SPE HLB C18) cartridge. The SPE cartridge was first activated with 3 ml methanol at a flow rate of 3 ml/min and then rinsed with 2 ml of double-distilled water. The centrifuged sample solution was loaded in an SPE cartridge at a flow rate of 5 ml/min. After loading each sample, the cartridge was treated with 2 ml of 5 % methanol solution in double-distilled water, the analytics were performed with 2 ml of HPLC grade methanol at a rate of 4 ml/min and the residue was resolved in 1 ml 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) of them had a positive reaction to the antibiotic residues. Table 1 gives the numbers of positive results on plates seeded with *B. subtilis* and *M. luteus*. On the plates seeded with *B. subtilis*, out of 47 samples positive for the presence of antibiotic residues, 13 (27.66 %) milk samples showed inhibition zone ≥ 2 mm on all 3 plates (pH 6, 7.2, and 8). Ten (21.28 %) samples were positive only on one of 3 plates (pH 6) and 14 (29.79 %) samples were positive only on two plates (pH 6 and 7.2).

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<
B. subtilis	6	199	26	21
	7.2	200	26	20
	8	212	25	9
	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

3.2. ELISA

After initial screening using the FPT, a semi-quantitative ELISA method was performed to detect the residue level of antibiotics in positive milk samples. The results of this analysis indicated that 29.79 % (14/47) of samples had a TC level higher than that of MRL, suggested by EU (100 µg/L). The average TCs residue in positive samples was calculated 98.43±6.86 µg/L. Additionally, the lowest and highest 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). Obtained validity parameters, including LOD, LOQ, Regression coefficient, and Retention Time (min) 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 revealed that 29.79 % of the samples were positive for TCs residues at levels above the defined MRL.

In HPLC analysis, all of the 14 positive samples in ELISA were contaminated with TCs. Among the TCs, the frequency and concentration levels of CTC were the most frequent, 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 average concentration of the three analyzed antibiotics among the positive samples (105.73 µg/L) was just a little bit 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).

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 samples	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

4. Discussion

A significant amount of antibiotics produced in the world 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 consumed foods that provides a large part of the daily needs of humans, especially for children (6). However, the adverse effects that can occur as a result of the use of veterinary drugs, as well as antibiotic resistance, are considered to be a major threat to human health. Therefore the food source must be free from any contamination (2). In the detection of drug residues, the 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 obtained in microbial assays can play a positive role in confirming the type of antibiotic available. The antibacterial properties of antibiotics or their metabolites in animals are variable and depend on the type of used compound 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. Also, these tests can be used to determine the type of antibiotic in the group detected by microbial tests (3). Observation of the inhibition zone around the samples in the FPT is only possible when the level of antibiotic residues is more than the

allowed limit. The sensitivity of FPT is high and cannot detect residues that are less than or within the permissible limits recommended by the EU. Changing the pH of the culture medium and the type of bacteria has the greatest impact on revealing the inhibitory effects of antibiotics in FPT (16).

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

In a study from Tehran, the capital of Iran, 8.7 % of pasteurized milk samples were positive for tetracycline and oxytetracycline residues (20). In Moghaddam et al (21) study from Northeast Iran, 28.7 % of milk samples were positive for 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 observed antibiotic residues (22). Regarding Nematiniko et al (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 report by Bahramian et al (1) from Iran, there were antibiotic residues in 26 % of raw milk and 21 % of pasteurized milk samples. In addition, this rate was detected in 28 %, 43 %, and 27 % by using screening, ELISA, and HPLC techniques, respectively (5). In a report from Brazil (22), the findings revealed that among the positive samples, 6 % were positive for both tetracycline and β-lactam antibiotics and 11 % were positive for beta-lactams, only. Consumption of milk containing antibiotics plays an important role in creating drug resistance. Proper withdrawal time of antibiotics can prevent the release of antibiotic residues in milk from livestock. Educating farmers on the risks of overusing drugs and antibiotics in livestock products is crucial to improving the current trend (3).

The highest diameter of the inhibition zone was observed at the lowest pH tested. This result shows the presence of tetracyclines, although beta-lactam antibiotics also behave in the same way. However, most beta-lactam antibiotics, especially penicillin, are less stable in storage conditions (2,3). Additionally, since tetracyclines were found in the majority of plates seeded with *B. subtilis*, the remaining semi-quantitative antibiotics were determined using a commercial ELISA kit for various tetracyclines. The pH of the culture medium, as previously indicated, has a significant influence on the outcomes. FPT cannot identify antibiotic residues below the MRL due to the

dispersion of compounds in the culture medium and that impact on the sample matrix. In the ELISA evaluation, 29.79 % of positive samples in FPT had antibiotic residues exceeding the maximum residue limit (MRL) set by the European Union for milk (100 µg/kg). The samples with tetracycline residue higher than MRL had an average level of 98.43±6.86 µg/L. In an ELISA investigation on antibiotic residues in 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 detected most frequently (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. 14.97 % of samples were lower than EU MRLs standards. Additionally, no significant differences were seen among the detection level of antibiotic residual and sampling seasons as well as 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 a variety of factors, including differences in the sample size, laboratory diagnostic methods, drug-using status, withdrawal times, type of monitoring, and health protocols in each area.

The presence of pharmaceutical residues in dairy products is unacceptable due to the harm it can cause to consumers and the negative impact on the food industry. Regarding our 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. There is no appropriate program or essential regulation on milk processing plants and milk collecting centres for evaluating antibiotic residues during milk delivery. It is recommended to train farmers on the correct use of drugs, especially antibiotics, and to observe the withdrawal period from milk according to the instructions of the farm veterinarian. It is also necessary to develop 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. Further research is necessary to identify potential programs that can reduce and control the concentration of contaminants in milk.

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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 on request from the corresponding author.

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