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Original Article

Assessment of Antibiotic Residues in Raw and Pasteurized Milk Produced by Different Analytical Methods

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Abstract
Background: Due to lack of attention to the required time of emulating the absorbed doses of drugs from different organs, inappropriate use of antibiotics for disease treatment in animals causes many adverse health effects in consumers**.** In this study, the antibiotic residues in raw and pasteurized milk were evaluated in Neyshabour city.
Methods: Raw and pasteurized milk samples were collected from eight farms and milk collection units and four pasteurized milk production factories in Neyshabour city during the warm and cold seasons. 56 samples of raw milk and 24 samples of pasteurized milk were analyzed using the Copan milk test, Tri-Sensor kit and ELISA detection methods to evaluate presence of the antibiotic residue.
Results: Copan milk test and Tri-Sensor kit did not detect the antibiotic residue. ELISA kit was able to detect the presence of gentamicin and streptomycin in milk. 25% of the samples contained gentamicin, while 10% of the samples contained streptomycin. The amount of antibiotic residue in the warm season was higher than cold season. 58.3% of pasteurized milk samples in the warm season had gentamicin.
Conclusion: Based on our results, extensive and accurate monitoring of antibiotics in raw and pasteurized milk needs to be performed in the city, especially in warm seasons.
Keywords: Antibiotics, Copan milk test, ELISA, Milk, Tri-Sensor

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Introduction

Dairy products as essential food sources contain necessary elements for a healthy diet.[1] Furthermore, milk is one of the most suitable and balanced food combinations[2] which is considered as a complete food. This compound contains large amounts of protein and all ten essential amino acids for body.[3] It also includes lipids, minerals, calcium and vitamins D, A, B_2 and B_{12}.[4] Milk has a vital role in the human food chain, especially for children. Therefore, according to quality criteria, it should be standard for consumers in terms physical, microbiological, chemical, and pharmacological properties.[5] Concerns about the safety of animal-source foods, such as milk have been increased through increasing urbanization, income growth, and lifestyle changes.[3] Therefore, milk is exposed to many contaminants through treating cattle, animals' diet, milking environment, and the factory's production process.[6] These contaminants include antibiotics, hormones, disinfectants, insecticides, and heavy metals.[7] The antibiotics belonged to a specific group of drugs are called antimicrobials which include penicillin, tetracycline, and amoxicillin. These drugs are used to remove or inhibit the growth of bacteria. Antibiotics can also affect the environment; they disrupt the sewage treatment process and microbial ecology of surface waters. In addition, in urban sewage treatment, they cause resistant prevail bacteria over other bacteria.[8]

The consumed drugs through injection, topical, or oral are somewhat accumulated in the body which is called antibiotic residue.[9] Over-use of antibiotics for treatment

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and prevention while lack of attention to the time required to dispose of the drug in animals results in health risks in consumers. Microorganism's resistance (especially pathogens), allergy in people who are allergic, elimination of beneficial organisms that are essential for preparing fermented milk products, and significant economic impact on the dairy industries are examples of the serious side effect.[10]

Mastitis is one of the most common diseases in cows, which is economically so important; it is one of the most costly dairy cows' flock conditions. The disease's adverse effects are reduction in milk production, discarding the milk of treated cows, and the use of antibiotics for treatment.[9] Various tests have been developed to detect antibiotics in milk. The proper method should be fast, cheap, simple, and suitable.[11] Moreover, the substances' effect on milk, their type, and sensitivity should be considered.[12] One of the methods is antibiotic test kits, a fast, specific, and applicable in production and monitoring centers.[13] The antibiotic residues in the food have adverse effects on the consumer. Therefore, milk quality and hygiene control in terms of these substances' residuals seem to be necessary.[13] The location and season of collected milk can affect the concentration of antibiotics in the product. Today, depending on the price, cost, and the experience of people working with kits, distinctive kits are used in different factories. However, some are not accurate enough. Therefore, comparing the accuracy of kits and introducing accurate ones can help improving the accuracy and precision of factories' results. So far, many researches on presence of antibiotics in milk have been conducted.[14-22] It has been reported that the use of kits can sometimes lead to false results. However, different kits are used to detect the presence of antibiotics in factories. The aim of this study was to determine different types of antibiotic residues in raw and pasteurized milk during warm and cold seasons in Neyshabour, the largest producer of milk in Iran. This research also aimed to determine pasteurization effects on reducing antibiotic residues in milk to introduce a fast and accurate method to milk factories and collection centers to detect antibiotic residues.

Materials and Methods

Given that amount of antibiotics varies from season to season, the tests were performed in August and February as warm and cold months of the year.[13] Samples were selected from eight farms and milk collection units and four pasteurized milk production factories in Neyshabour city. Sampling was from raw and pasteurized milk. We did the sampling three times per season in milk factory and two times per season in milk collection units. 28 samples of raw milk and 12 samples of pasteurized milk were tested. Given that the samples were tested in both warm and cold seasons, total samples of this study were 80 different milk samples. Sampling was performed in full compliance with the sampling principles. Thus, we did the sampling in hygienic conditions with clean and suitable equipment. A sample of 500 cc was taken from the discharge valve of the tanker containing milk. Furthermore, if the milk was in the milk tank (Bidoon), it was thoroughly mixed with a steel spoon before sampling. The sample was poured into a particular container and closed with a lid. All the samples property, including sample description, location, sampling date, etc., were prepared for testing and transferred to the veterinary laboratory of Khorasan Razavi province under cold condition. The sample was hold on a shaker in the laboratory until it reached room temperature and became utterly homogeneous. Sample dishes with their own number were placed on the laboratory platform and tested based on the hygienic principles. In this study, Copan Milk Test (CMT) (ECLiPS50, Spain), aminoglycosides enzyme-linked immunosorbent assay (ELISA) (Tecna, Italy), and Tri-Sensor (Unisensor Tri-Sensor, Belgium) were used to detect antibiotics in milk. Finally, High-performance liquid chromatography (HPLC) (Agilent Technologies, Part No:5982-5550, USA) was used to determine the positive samples in terms of antibiotic concentration. The CMT primarily detects the antibiotic residues in milk.[23] It can see values above the maximum residue limits of the antibiotic and which is stored at a temperature of 4 to 12 degrees. After shaking the sample container, 50 Landa of milk samples were poured into the kit. It was then incubated at 65°C for 2 hours and 15 minutes or 2 hours and 45 minutes. When the control color changes to yellow, it is an excellent time to leave the incubator. After leaving the incubator, the wells were washed with distilled water for 2 to 3 times. The yellow and blue or bluish colors represent negative and positive results, respectively. ELISA kit is used to detect streptomycin and gentamicin in milk. ELISA was utilized as a diagnostic tool. The ELISA diagnostic reaction was performed as follows; first, the specimens were completely homogenized by the vortex. Then, 20 μL of standard gentamicin solutions, test specimens, and diluent solutions were added to the respective wells. Afterwards, 100 μL of the solution was added to each well and thoroughly mixed by shaking the microplate with rotating motions for a few seconds. The microplate was incubated for 20 minutes at condition of room temperature and away from light. Then, the microplate wells' contents were emptied, and the wells were washed three times by an automatic washing machine. The remaining droplets sticked on the internal wells' surface were entirely removed by tapping the microplate on several dry paper towels. In the next step, 120 μL of the substrate and chromogen as the color development was added to each well. After rotational shaking, the microplate was incubated for 25 minutes at 25°C and away from light. At the last step, the color reaction was investigated by adding 50 μL of the inhibitory solution to wells. Therefore, the blue color changed to yellow. The plates were in read with an ELISA reader (BIOTEK Instruments) at a wavelength of 450 nm.[6] The Tri-Sensor kit was similar to the ELISA kit. It was used to detect the tetracycline antibiotics, Sulfonamides, and gentamicin in milk.

The data were analyzed using analysis of variance (ANOVA) and Tukey test by Minitab software. The statistical significance was considered as $P < 0.05$.

Results and Discussion

The diagnostic result of CMT showed that only one sample (among 80 samples) contained antibiotics that belonged to the milk collection station. The results of Tri-Sensor kit showed that among the total samples only one sample contained beta-lactam and one sample contained sulfonamides, which were belonged to milk collection stations. Based on results of this kit, tetracycline was not detected in the samples. In a study conducted by Movassegh[24], ELISA was used to investigate the antibiotic residues of milk. The authors found that out of 50 collected milk samples, 24% contained antibiotic residues. Therefore, it can be said that this kit was more accurate than the CMT and Tri-Sensor kit for detecting antibiotics. It is reported that ELISA is a suitable method for determining aminoglycosides, including gentamicin in milk.[25] The results of ELISA for gentamicin and streptomycin antibiotics are given in Tables 1 and 2, respectively. Table 1 shows that 75% of the samples did not have gentamicin antibiotics, and 25% of them were positive by the ELISA test. The highest level of contamination was between 1.26-2.5 μg/L. The highest amount of gentamicin antibiotic was found in the warm season. According to the ELISA results, 47.5% of the samples collected in warm season contained antibiotics, while only 2.5% of the samples contained antibiotics in the cold season. As seen in Table 1, the highest level of contamination in the warm season was observed in raw milk factories. The lowest level of contamination in the cold season was found in pasteurized milk and raw milk collection centers. Milk collection had no concentration of gentamicin. The results of comparison between the number of contaminated samples of raw and pasteurized milk in warm and cold seasons revealed that there was a significant difference between samples containing gentamicin; the number of contaminated samples in the warm season was higher than the cold season ($P < 0.05$). Also, the results of comparison between the contamination rate of different samples of raw and pasteurized milk in warm and cold seasons revealed that there was no significant difference between gentamicin's residues ($P > 0.05$).

The statistical analysis showed no significant difference between sampling sites in terms of contamination ($P > 0.05$). The contamination of milk samples in the warm season was higher than similar samples in the cold season, which can be due to higher prevalence of mastitis in warm seasons. As a result of taking antibiotics and not observing the period of abstinence, the antibiotic residues in milk collected in warm season are more than the cold season. According to other researches, the ELISA method has the highest speed and sensitivity for measuring antibiotics. Also, this method is economically viable.[6] Raza et al[18] used HPLC and ELISA kits for investigation of penicillin and oxytetracycline residue in raw, UHT and pasteurized milk of buffalo and cows. Kit and HPLC method were used to identify antibiotic residues. Raw milk of buffalo and cow collected from the open market have the highest positive rate (23.5%), and UHT milk has the lowest positive rate (8.5%). Joubrane et al[26] measured the antimicrobial residues and antimicrobial resistance in raw bovine milk in Lebanon. Approximately, 195 samples of raw milk during 2 years were collected by the authors. Their results showed that antimicrobial residues of oxytetracycline, tetracycline, ciprofloxacin, sulfamethazine, and doxycycline were below the maximum residue limits, and there was a high level of antimicrobial resistance in the sampled milk.

Other studies conducted in different country have found different antibiotic contamination in different sampling time by different diagnosis methods. Contamination rate of raw milk in Mashhad,[6] Shiraz,[27] Kerman,[28] and Sarab, Ardabil[24] were 11.76%, 2.75%, 67.5%, 14%, respectively. Regarding pasteurized milk, Tetracycline contamination rates in Tehran was between 7.1% and 7.8%.[9,29] Ghanavi investigated the contamination of antibiotics at the milk

Table 1. Residual amount of Gentamicin in Milk Samples Collected in Hot and Cold Seasons

ELISA Test for Gentamicin	Source of Sampling	Tested Sample	Positive Samples	Negative Samples	Distribution of Positive Samples in the Standard Range (μg/L)				
					0-0.625 0.3	1-0.25 0.63	2-0.5 1.26	5-2.51	5 >
Warm season	Raw milk (milk collection unit)	16	1	15	0	0	0	1	0
	Raw milk (factories)	12	11	1	0	0	11	0	0
	Pasteurized milk	12	7	5	0	4	2	1	0
	Total (%)		47.5	52.5	0	10	32.5	5	
Cold season	Raw milk (milk collection unit)	16	0	16	0	0	0	0	0
	Raw milk (factories)	12	1	11	0	0	1	1	0
	Pasteurized milk	12	0	12	0	0	0	0	0
	Total (%)		2.5	97.5			2.5	2.5	
Total (%)			25	75	0	5	17.5	3.75	0

Table 2. Residual Amount of Streptomycin in Milk Samples Collected in Warm and Cold Seasons

ELISA Test for the Diagnosis of Streptomycin	Sample Source	Tested Sample	Positive Samples	Negative Samples	Distribution of Positive Samples in the Standard Range (µg/L)				
					0-0.625 0.3	1-0.25 0.63	2-0.5 1.26	5-2.51	5 >
Warm season	Raw milk (milk collection unit)	16	3	13	0	1	1	1	0
	Raw milk (factories)	12	1	11	1	0	0	0	0
	Pasteurized milk	12	3	9	0	0	3	0	0
	Total (%)		17.5	82.5	2.5	2.5	10	2.5	0
Cold season	Raw milk (milk collection unit)	16	0	16	0	0	0	0	0
	Raw milk (factories)	12	1	11	1	0	0	0	0
	Pasteurized milk	12	0	12	0	0	0	0	0
	Total (%)		2.5	97.5	2.5	0	0	0	0
Total (%)			10	90	2.5	1.25	5	1.25	0

produced in the factories using Delvo, CMT, Beta Star test, and Cylinder-plate method. The results showed that 27% of raw milk and 53% of pasteurized milk was contaminated.[30] According to the EU standard, the allowable maximum residue of gentamicin in milk is 100 µg/mL, and based on the FAO/WHO' standard, it is 200 µg/mL. Gentamicin's maximum allowable residue is 50 µg/L and 100 µg/L in cow's milk according to US and Europe' laws, respectively. The acceptable daily intake of gentamicin is 4 µg/kg for an individual or 240 µg per person.[6] As shown in Table 1, the amount of gentamicin detected in the milk in Neyshabour city was less than other cases. It has been reported that after injection of gentamicin and its appearance in milk, its amount decreases over time due to renal excretion. Because the exact time of injection is unknown and because of the increase in the dilution of gentamicin in the storage tank of milk, it can be concluded that the concentration of gentamicin in livestock is higher than the amount obtained in this study.[6] Fallah-Rad et al[6] investigated the amount of gentamicin in raw milk delivered to Mashhad pasteurized milk factory and pasteurized milk obtained from the same raw milk. The results showed no significant difference between the amount of gentamicin in raw and pasteurized milk. Our results were consistent with the results obtained by the researchers. The results showed that 58.3% of pasteurized milk samples in the warm season contained gentamicin (Table 1). The high level of antibiotic contamination in pasteurized milk can be explained by use of healthy raw milk without antibiotics in dairy factories, which is usually used to prepare fermented dairy products such as yogurt and cheese. To prepare the sterilized milk (ultra temperature), the high quality raw milk is used to enable a high thermal process. Finally, contaminated and low-quality milk is used to produce pasteurized milk. As a result, contaminated and low-quality milk is mainly consumed by the general public. Therefore, it is necessary to adequately monitor the presence of antibiotics in the milk of farms and pasteurized milk factories.[13] As shown in Table 2, only 10% of the samples contained streptomycin, which similar to the gentamicin it was in the range of 1.2-2.26 ng/mg. The highest streptomycin contamination of the milk in collection unit was found in the warm season. It was between 1.5-2-2.5 ng/mg (10%). The lowest contamination was related to the same milk samples in the cold season (i.e., no antibiotic was detected). Similar to gentamicin's results, a significant difference was observed between the number of samples containing streptomycin in warm and cold seasons. In the study, the number of infected samples in warm season was higher than the cold one ($P<0.05$). Also, in terms of infection rate, no significant difference was observed between the residual amount of streptomycin in cold and warm seasons ($P>0.05$). Besides, no significant difference was observed between different sampling sites ($P>0.05$). Mahmoudi et al[2] measured the antibiotic residues in raw milk in warm and cold seasons. They found that 43% of raw milk samples contained antibiotics. and the CMT kit was not accurate enough to detect the antibiotics. ELISA kit was able to detect gentamicin and Sulfonamides. The concentration of antibiotics in the warm season was significantly higher than the cold season. Bahramian et al[22] conducted a systematic review and meta-analysis, and reported that the antibiotic residues in raw and pasteurized milk in Iran was 26% and 21%, respectively. They also showed that permanent control of milk in the collection centers by the responsible organizations along with implementation of Hazard Analysis Critical Control Point (HACCP) system in milk factories is very important for reduction of antibiotic residues in milk.

Conclusion

The CMT and Tri-Sensor kits could not detect the antibiotics in milk. ELISA kit was able to detect gentamicin and streptomycin in milk. 25% of the samples contained gentamicin, and 10% of them had streptomycin. The highest infection rate was related to the warm season. It might be due to increased prevalence of diseases, especially mastitis, and increased use of antibiotics. The highest rate of gentamicin contamination was related to the factory's raw milk. Also, the highest rate of streptomycin contamination was related to pasteurized and raw milk of collection units. There was no significant difference between the samples in terms of gentamicin and streptomycin antibiotics, and their level were lower than the standard value. A relatively large volume of pasteurized milk contained antibiotics, which can be explained by low-level use of milk to produce pasteurized milk. Due to consumption of pasteurized milk by the general public, extensive and accurate monitoring of milk quality, especially in the warm season should be done.

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Competing Interests
The authors declare that they have no competing interests.

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