Detection of antibiotic residues in locally raw milk by using high performance liquid chromatography at different seasons and the effect of heat treatment on their concentration

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Summary

The aim of this study was to determine the level of antibiotics residues in the locally produced cows raw milk as well as to evaluate the effect of different commercial heat treated process on the level of antibiotics residues in milk. A total of 52 locally produced milk samples were collected from apparently healthy cows in AL-Fudhailia village, milk cans (50 Kg), bulk milk tanks (1, 5 and 10 tons) which belonged to the milk reception and collection centers and dairy plants in Baghdad were collected during beginning of December 2015 to the end of April 2016. Results revealed by using high performance liquid chromatography that there was a significant seasonal variation in the residual concentration of each detected antibiotic in milk samples, where it was found that all cow's milk samples had significantly higher concentrations of antibiotic residues in spring than in winter seasons. Generally by excluding both the season and the kind of antibiotic, it was found that milk samples that were collected from 50 Kg milk cans recorded significantly highest antibiotic residues followed by bulk milk tanks of 1 and 5 tons in comparison to 10 tons. Antibiotics recovery experiment was conducted by spiking the bovine milk samples with Known concentrations four ß-lactams (Benzylpencillin, Cloxacillin, Amoxcilin, Ampcilline) and other five detected antibiotics and then exposed to one of the three different commercial heat treatments. The results showed that the pasteurization process (63°C/30 min.) a slight inactivation of four ß-lactams and other five detected antibiotics, whereas 80°C/5 min. high degree of antimicrobial loss at the rate of 89% and 82%. However, boiling at 100°C/5 min, high degree of antimicrobial loss at the rate of 100% and 90% respectively.

Keywords: High performance liquid chromatography, Antibiotics, Raw milk.

Introduction

Antibiotics are the most widely used veterinary drugs for the therapeutic and prophylactic purposes and also as growth promoter in dairy animals which may appear in milk as residues for a certain time period (1). The usage of antibiotics in excess amount may lead to have residues in milk following 12-96 hours after the injection based on a variety of factors (2). The antibiotic contamination of milk was reported to be due to the intramammary infusions of antibiotics for treating mastitis (92%), injections (6%), and other causes 2% (3). Antibiotic residues in food are potential threat to direct toxicity in human and their low levels would result in death of intestinal flora, cause disease and possibly develop resistance strains which cause failure of antibiotic therapy in clinical situations. Other harmful effects related to antibiotic residues in food were allergic reactions anemia, disturbances in stability of the gut microflora, and antibiotic resistance and its transfer to the human (4). The presence of antibiotic residues in milk was considered primarily a manufacturing problem related to inhibition of dairy starter microorganisms and cause economic losses in cheese and fermented milk industries (5). The presence of residues may be the result of failure to monitor the withdrawal periods, illegal or off-label use of drugs and incorrect dosage levels or dosing schedule. Unauthorized antibiotic use and lack of good veterinary practices may result in residues of these substances in milk and tissues (6). The World Health Organization (WHO) and Food and Agriculture Organization (FAO) have set certain standards for acceptable daily intake and maximum residues limit (MRLs) for each antibiotic in

milk. The increasing awareness of food safety by the consumer with respect to antimicrobial resistance has resulted in increasing pressure on laboratories responsible for food safety to monitor the use of these drugs and ensure the safety of food (7). To detect specific antibiotics present in very low quantities high performance liquid chromatography (HPLC) technique which was considered to be the most precise method was used in the current study. In this study, the aims were to determine the levels of antibiotics residues (ppb) in the locally produced cow's raw milk as well as to evaluate the effect of different commercial heat treatments process such as 63°C/30 min., 80°C/5 min. and 100 °C/5 min. on the levels of antibiotic residues in milk.

Materials and Methods

All raw milk samples were collected in a clean sterile 500 ml polyethylene plastic bag and examined during two climatic periods. Each polyethylene plastic bag was supplied with a written label which specified the product's name, and any other useful information for further identification. The samples were conserved cooled in a portable ice cooled box during the transfer to the laboratory of the Veterinary Public health department. Each raw milk sample was divided into two portions where the first portion was sent to the HPLC analysis to determine the levels (ppb) of antibiotic residues in milk while the other portion was stored inside the refrigerator at 4°C until the heat treatment process was conducted in the laboratory. Some of the positive samples for the antibiotic residues were selected randomly and subjected to one of the commercial heat treatment processes such as 63°C/30 min., 80°C/5 min. and 100 °C/5 min. to determine the effect of heat treatment on the levels of antibiotic residues in milk.

Milk samples were distributed as following: cow's raw milk samples were collected during the first period (December 2015, January 2016). Twelve of locally produced raw cow's milk samples were collected from bulk milk tanks of different capacities such as 1 ton, 5 tons and 10 tons (4 samples /tank) which belonged to various dairy cattle station, milk reception and collection centers, and dairy plants in Baghdad province. Four of locally produced raw cow's milk samples were collected from milk cans of 50 Kg milk capacity inside the producer's homes at Al-Fudhailia village. Ten milk samples were collected from the apparently healthy individual cows inside the producer's homes at the Al-Fudhailia village.

Cow's raw milk samples were collected during the second period (March, April 2016). Twelve of locally produced raw cow's milk samples were collected from the bulk milk tanks of different capacities such as 1 ton, 5 tons and 10 tons (4 samples /tank) which were belonged to various dairy cattle stations, milk reception and collection centers. Four of locally produced raw cow's milk samples were collected from milk cans of 50Kg milk capacity inside the producer's homes at Al-Fudhailia village. Ten milk samples were collected from the apparently healthy individual cows inside the producer's homes at the Al-Fudhailia village. Three cow's raw milk samples that were positive for the presence of antibiotic residues were selected randomly and subjected to one of the commercial heat treatments process such as 63°C/30 min., 80°C/5 min. and 100°C/5 min. to determine the effect of the heat treatment for the degradation of antibiotic residues in milk (Three replications for each treatment). The milk samples were collected and prepared for quantitative analysis using HPLC with solvent A (Acetonitrile) and solvent B (20 Mmkh2 PO4, pH 3.5) as mobile phase. The standard of active material was separated on Fast Liquid Chromatographic (FLC) column, linear gradient from 0%b-100% B in 12 min. Detection UV set at 220 nm. Temperature ambient. Flow rate 1.5 ml/ min. injection volume 20 ul. The separation occurred on liquid chromatography Shimadzu 10AV-LC equipped with delivery pump model LC-10A shimadzu, the eluted peaks were monitored by UV-Vis 10A-SPD spectrophotometer (8-10)

Milk samples were analyzed using the liquid chromatography system developed by (11). A chromatography Column, nucleodur C-18 gravity, 5 u particle size (150 X4.0 mm I.D) column was used. UV spectrophotometer detector was carried out at the wavelength of

220 nm. The measurements took place in isocratic mode.

Results and Discussion

The Results of seasonal variation in the mean levels of antibiotics residues in milk sample collected from the individual cows in Al-Fudhailia village were illustrated in (Table, 1) which establish the statistically significant (P≤0.05) influence of the season on the concentration of each detected antibiotic residues. It was found that each of the four detected antibiotics such as Benzylpencillin, Oxytetracycline, Sulfonamide and Cloxacillin had significantly (P≤0.05) higher residual concentrations in spring than in winter seasons. While there were no significant differences in the Gentamycin residues between these seasons. The results of seasonal variation of the mean levels of residual concentration for each of the eight different antibiotics in milk samples collected from all milk storage cans and tanks of different capacities are shown in (Table, 2). The laboratory studies of the residual antibiotics investigation using the HPLC technology during the two climatic periods (winter and spring seasons) of the study revealed that there was a significant (P≤0.05) seasonal variation in the residual concentration of each antibiotic where the average mean values of Sulfonamide, Gentamycin, Cloxacillin, Rifampicin, Amoxicillin and Ampicillin in bovine raw milk samples collected from all the milk storage cans or tanks of different capacities were 8.369±0.57, 3.225 ±0.12, 11.113±0.08, 0.0, 15.677±4.62 and 2.941±0.05 ppb respectively, in spring season whereas were 3.432±0.09, 1.247±0.06, 2.614 ±0.06, 1.952±0.04, 1.029±0.02 and 0.0 ppb respectively in Winter season (Table, 2).

These results were in agreement with (12) who reported an increasing number of samples contaminated with antibiotic residues was found in late winter - early spring with a maximum in April but by early summer there was a decrease in the number of contaminated samples and during the last months of summer-early autumn there was a further increase in the number of contaminated samples. The seasonal variations recorded for antibiotic contamination of milk was considered an indirect consequence of seasonal calving system adopted in the North Eastern Romania. Thus, due to the prolonged use of grazing, most births were scheduled for spring (February – March). Because of this, in spring occured an immunity decrease on the general fund of post-partum stress associated with the scarcity of food. Anothers (١3) found that the β-lactams antibiotic residues in milk samples collected during winter season were higher than the samples collected during summer season, because dairy cattle was the most frequent reason for mastitis treatment.

This result was supported by (١4) who found that the milk samples collected in summer showed higher percentage (25%) to those which were collected in winter season (7.9%), which indicated effects of higher temperature on bacterial growth. While (١5) reported that the prevalence of antibiotic contaminated milk samples was raised in the warm season of the year which could be attributed to the higher incidences of diarrheal diseases in the cattles which result in the enhancement of antibiotic administration to the cattle.

Table, 1: Comparison the mean levels of antibiotic residues (ppb) in milk samples collected from the individual cows in AL-Fudhailia village between the winter and spring seasons.

Type of the detected antibiotic	No .of examined samples per season	% Frequency	Winter	Spring	LSD
			Mean ± SE	Mean ± SE	
Benzylpencillin	10	30	0.542 ± 0.06 B	2.293 ± 0.06 A	0.562 *
Oxytetracycline	10	35	3.494 ± 0.09 B	10.312 ± 0.71 A	2.189 *
Sulfonamide	10	20	2.753 ± 0.04 B	5.060 ± 0.24 A	1.763 *
Gentamycin	10	20	0.753 ± 0.05 A	0.725 ± 0.05 A	0.337 NS
Cloxacillin	10	20	1.938 ± 0.06 B	3.438 ± 0.09 A	0.654 *

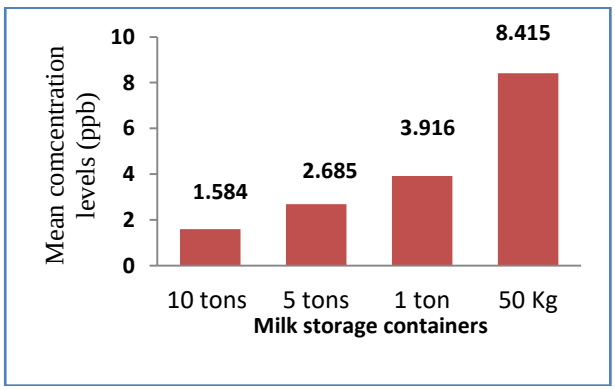
*Horizontal different capital letters revealed a significant (P<0.05) differences between the seasons.

Table, 2: Seasonal variation of the mean levels of residual concentration (ppb) for each of the eight different antibiotics in milk samples collected from all milk storage tanks.

Type of the detected antibiotic	No .of examined samples per product	Winter ppb	Spring Ppb	SD
		Mean ± SE	Mean ± SE	
Benzylpencillin	16	3.065 ± 0.13 A	4.322 ± 0.09 A	1.278 NS
Oxytetracyclin	16	4.058 ± 0.29 A	5.273 ± 0.21 A	1.309 NS
Sulfonamide	16	3.432 ± 0.09 B	8.369 ± 0.57 A	2.184 *
Gentamycine	16	1.247 ± 0.06 B	3.225 ± 0.12 A	1.073 *
Cloxacillin	16	2.614 ± 0.06 B	11.113 ± 0.08 A	2.226 *
Rifampicin	16	1.952 ± 0.04 A	ND B	1.632 *
Amoxcilin	16	1.029 ± 0.02 B	15.677 ± 4.62 A	7.529 *
Ampcilline	16	ND	2.941 ± 0.05	-----
Total		2.485	7.274	

*** Horizontal different capital letters revealed significant (P<0.05) difference between seasons.**
***ND=Not Detected**

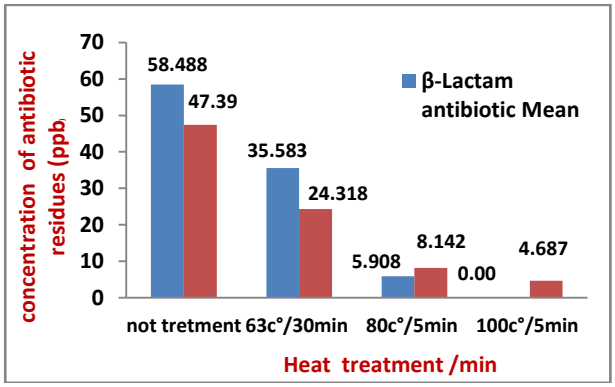
Comparison of the mean levels (ppb) of different antibiotic residues between milk samples collected from milk cans and bulk milk tanks of different capacities for both seasons is shown in (Fig. 1). Generally by excluding both the season and the kind of antibiotic, it was found that milk samples that were collected from 50Kg milk cans recorded significantly (P≤0.05). The highest antibiotic residues (8.415 ppb) followed by bulk milk tanks of 1and 5 tons where their mean levels of antibiotic residues were 3.916 and 2.685 ppb respectively in comparison to milk samples that were collected from the 10 tons bulk milk tank that had significantly (P≤0.05) the lowest concentration of antibiotic residues with the mean level of 1.584 ppb as shown in (Fig. 1).



Figure, 1: Differences in the mean levels of all eight different antibiotics residues between milk containers of different capacities for both seasons.

The extensive bulking of milk from multiple sources associated with milk retailers may have reduced the concentration of antibiotic residues to below the threshold levels (16) might be due to the dilution effect of milk bulking, researchers (١7) reported that the high percentage of antimicrobial residues in raw milk collected from vendors might be due to the adulteration by addition of the antibiotics to the milk as preservatives to prolong its shelf life. The results of efficacy of different commercial heat treatments on the degradation of the antibiotic residues in bovine milk are shown in (Fig. 2). The results of the current study revealed that there was a significant (P<0.05) effect of exposing the bovine milk that fortified with nine different antibiotics to the three different heating temperatures for different exposure times on the concentrations of both the β-lactams (Benzylpencillin, Cloxacillin, Amoxcilin and Ampcilline) and the other antibiotics such as Oxytetracycline, Sulfonamide, Gentamycine, Rifampicin and streptomycin. The results showed that Vat pasteurization at 63°C/30 min. in the laboratory provoked a slight inactivation at the rate of 39% (35.584 ppb recovery) for all the four β-lactams antibiotics but somewhat higher inactivation at the rate of 48% (24.318 ppb recovery) for all the other five different antibiotics. In contrast, all of the four β-lactams antibiotics underwent a high

degree of antimicrobial loss at the rate of 89% (5.908 ppb recovery) when the bovine milk samples were subjected to 80°C/5 min. and similar reduction in the activities of the other antibiotics was obtained where the inactivation percentage from 80°C/5 min. was 82% (8.142 ppb recovery). However, boiling of the bovine milk samples in the laboratory at 100°C/5min. led to the highest inactivation percentage (100%) in β-lactams antibiotics, while produced a marked high degree of inactivation in the other antibiotics where the inactivation percentage was (90%) (4.687 ppb recovery) as shown in (Fig. 2).



Figure, 2: The percentages of thermal inactivation of the β-lactam and other antibiotics residues in bovine milk.

Worker (18) indicated that milk samples with beta-lactams antibiotics reached low percentages of inactivation (80%) for milk pasteurization at 62.8 °C/30 min., also similar reduction in the activity of Oxytetracycline (23.6%) was obtained. Previous studies suggested that Cloxacillin was heat stable while Penicillin G, Ampicillin and Amoxicillin were appeared partially heat labile (19). In general, the beta-lactams antibiotics are stable when heated at low temperature such as those used in the laboratories at 63°C/30 min. Overall, the data of the current study confirmed the published results which showed that the Vat pasteurization (63°C/30 min.) led to partial reduction in the concentrations of all antibiotics residues in milk but did not cause complete degradation of such substances. Higher reduction in the antibiotics obtained with heating at higher temperature as shown in other studies (20) which indicated that the Benzylpencillin was stable at 65°C but not stable at higher temperatures. Boiling of the bovine milk samples for 5 minutes offered an effective approach since it greatly reduced the levels (ppb) of antibiotics residues to less than that recommended by the WHO and FAO.

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الكشف عن متبقيات المضادات الحيوية في الحليب الخام المحلي والمستورد باستعمال تقنية كروموتوكرافي السوائل عالي الأداء خلال فصول مختلفة وتاثير المعاملات الحرارية في تركيزها

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الخلاصة

الهدف من الدراسة هو الكشف عن وجود متبقيات المضادات الحيوية في الحليب الخام المنتج محلياً ودراسة تأثير المعاملات الحرارية في مستوى تلك المتبقيات. جُمعت ٥٢ عينة من الحليب الخام المحلي جمعاً عشوائياً من أبقار محلية والتي تبدو بصحة جيدة من قرية الفضيلية ومن الحاويات المعدنية للحليب الخام سعة (٥٠ كغم) فضلاً عن الخزانات الرئيسة للحليب سعة (١ و٥ و١٠ طن) من مراكز جمع الحليب ومعامل الألبان في مدينة بغداد منذ شهر كانون الأول ٢٠١٥ لغاية نهاية شهر نيسان ٢٠١٦. كشفت الدراسات المختبرية باستعمال كروماتوكرافي السوائل عالي الأداء في مدتي الشتاء والربيع من هذه الدراسة أن هناك فرقاً معنويا اعلى في تركيز متبقيات المضادات الحيوية في الحليب في الربيع عن الشتاء في عينات الحليب التي جُمعت من الأبقار الفردية في قرية الفضيلية كذلك كانت القيم المتوسطة من متبقيات المضادات الحيوية في عينات الحليب التي جُمعت من الحاويات المعدنية سعة ٥٠ كغم وخزانات ذات سعات مختلفة أكثر في موسم الربيع منها في موسم الشتاء. كما وجد تاثير لسعة الخزان في تركيز متبقيات المضادات الحيوية بغض النظر عن الموسم ونوع المضاد الحيوي حيث أن عينات الحليب التي جُمعت من الحاويات المعدنية سعة ٥٠ كغم سجلت أعلى تراكيز لبقايا المضادات الحيوية تليها خزانات الحليب سعة ١ و٥ طن على التوالي، أما عينات الحليب التي جُمعت من خزان ١٠ طن فقد سجلت أقل تراكيز لبقايا المضادات الحيوية. كما أجريت دراسة تجريبية لمعرفة تاثير المعاملات الحراريه المختلفة في تسعة أنواع من المضادات الحيوية معروفة التراكيز في حليب الأبقار، أربعة منها مجموعة بيتا لاكتميز (بنزيل بنسيلين وكلوكساسلين واموكسيسلين وامبيسلين) وخمسة مضادات أخرى. حيث أظهرت النتائج أن درجة حرارة البسترة (٦٣°م/ ٣٠ دقيقة) لها تاثير قليل على مجموعة بيتا لاكتميز الأربعة واكثرعلى الخمسة الأخرى من المضادات الحيوية في حين أدت درجات الحرارة العالية (٨٠°م/ ٥ دقيقة) إلى حدوث انخفاض في المضادات الحيوية بلغت ٨٩% و٨٢% بالنسبة إلى مجموعة بيتا لاكتميز والمضادات الأخرى على التوالي في حين أظهرت درجة الحرارة (١٠٠°م/ ٥ دقيقة) انخفاضاً ١٠٠% في مجموعة بيتا لاكتميز و ٩٠% بالنسبة إلى باقي المضادات.

الكلمات المفتاحية: كروموتوكرافي السوائل عالي الأداء، المضادات الحيوية، الحليب الخام.