Detecting Antibiotic Residues Among Sheep Milk using YCT, DDA, and Acidification Method in Erbil city, Kurdistan Region, Iraq

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Abstract

Presence of antibiotic residues in milk is increasingly recognized as a global health issue owing to associated health risks and emergence of antibiotic resistant bacteria. The aim of this study was to determine the prevalence of antibiotic residues in sheep raw milk sold in Erbil governorate. The screening tests performed were; yoghurt culture, acidification, and disc diffusion tests. Randomly selected farms and sale points within the city were sampled. Out of 450 collected samples, 15.8% and 14.4% of them contained antibiotic residues according to yoghurt culture and disc diffusion tests, respectively. However, acidification test showed insignificant (P = 0.384) slightly higher prevalence (17.3%). Minor association was found between increase of residue prevalence and progress of summer-autumn months. Raising awareness among farmers and sale workers regarding withdrawal periods and risks of antibiotic residues are highly recommended to mitigate the health issues associated with consumption of contaminated milk.

Keywords: screening, YCT, DDA, acidification, Kurdistan region, Erbil, Iraq.

Introduction

Antibiotics are chemically diverse organic compounds with low to medium molecular weight exhibiting a variety of antibacterial properties. Antibiotics are extensively used in modern medicine of both human and veterinary disciplines. Almost all the classes of antibiotics that are used for humans are also used for food producing animals (Nirala *et al.*, 2018; Savarino *et al.*, 2020). Globally, antibiotics are used in food producing animals for therapeutic, prophylactic, metaphylactic, growth enhancement, and weight gain purposes. In such cases, the antibiotics are administered in the drinking water or feed (Yadav & Kumar, 2020; Rasschaert *et al.*, 2020).

Antibiotic residues term refers to the molecules that remain in food of animal origin for certain periods of time (Aidara-Kane *et al.*, 2018; Stella *et al.*, 2020). Lack of awareness among breeders and farmers regarding the withdrawal periods and health risks associated with residues contamination, especially in developing countries, are globally recognized issues (Garcia *et al.*, 2019; Rana *et al.*, 2019). Additionally, failure to follow the instructions of antibiotics manufacturers also accounts for residues occurrence in milk (Bacanlı & Başaran, 2019; FSA, 2015). The withdrawal period can be defined as the time that must elapse between the last administration of a veterinary medicine and the slaughter or production of food from that animal, to ensure that the food does not contain levels of the medicine that exceed the maximum residue limit (Anika *et al.*, 2019; El-Makarem *et al.*, 2020). Indeed, the presence of residues at limits exceeding the acceptable range is considered illegal (Sachi *et al.*, 2019).

Various negative health consequences have been identified due to the presence of antibiotic residues in foodstuffs including milk (Jayalakshmi *et al.*, 2017). Emergence of antibiotic-resistant bacteria, failure of starter culture during fermentation processes, carcinogenesis, mutagenic effect, and congenital anomalies due to prolonged exposure to residues during gestational periods are the most prominent health risks (Maharjan *et al.*, 2020). Antimicrobial metabolites have also been found to be transformed back to their original active form under certain environmental conditions (Manzetti & Ghisi, 2014).

Global consumption of sheep milk and its derivatives is massive because milk is a major source of high-quality proteins and fats (Balthazar *et al.*, 2017; Barros *et al.*, 2020). It also contains the required nutrients such as calcium, magnesium, selenium, riboflavin, vitamin B12 and pantothenic acid (Górska-Warsewicz *et al.*, 2019; Scrafford *et al.*, 2020). However, in recent years, the frequency of occurrence of veterinary drug residues in food, especially milk, has become an alarming issue (Almashhadany, 2009; Moghadam *et al.*, 2016; Olatoye *et al.*, 2016; Al-mashhadany *et al.*, 2018; Al-mashhadany, 2019). To the best of our knowledge, no study has been done to determine the antibiotic residues among milk sold in Erbil governorate. Therefore, the objective of this study was to determine the frequency of antibiotic residues in raw sheep milk at Erbil governorate using different assays. The association between months and detection of antibiotic residues among sheep milk samples was also investigated.

Materials and Methods

Study design and sampling

During the period from July to December 2019, a total of 450 raw sheep milk samples (230 from farms and 220 from sale points) were collected randomly and aseptically as described previously (Almashhadany & Osman, 2019). Farms were in suburban areas while the sale points were in different retail markets inside Erbil governorate. The collected samples were placed in separate sterile bottles (50 mL), and transported to Department of Medical Lab Science at Knowledge University (Erbil, Kurdistan Region, Iraq) under chilling condition.

Preparation of bacterial spores and test plates

Spores suspension of *Bacillus subtilis* (obtained from the Center for Academic Research, Training and Activities (CART), Knowledge University, Erbil city) was prepared according to standard method (Al-mashhadany *et al.*, 2018). Concisely, heavy inoculum of *B. subtilis* were introduced onto Nutrient agar plate (HiMedia, India) and incubated aerobically at 30°C for 10 days. After the incubation period, colonies were harvested into 10 mL of sterile normal saline and heated at 70°C for 10 minutes to kill the vegetative cells. Three cycles of re-suspension in sterile water and centrifugation at 1500 x g for 10 minutes were performed to obtain a purified spore suspension. Muller-Hinton agar was prepared as recommended by the manufacturing company (HiMedia, India). After cooling to 45°C, the medium was seeded by a volume of 0.1 mL of spore suspension to each 100 mL of the agar before solidification. After thorough mixing, the molten agar was poured into Petri dishes and allowed to solidify at room temperature. Plates were used at the same day of preparation.

Detection of antibiotic residues

Yoghurt culture test

Yoghurt culture test was done by lowering the pH of 96 mL of the tested milk to 6.0 pH using 1 N HCI followed by adding 4.0 gm of yoghurt culture and 1 mL of the pH indicator chlorophenol red (0.2% in 50% ethanol). The mixture was incubated at 42°C for 3 hours. Negative result (absence of antimicrobial residues in the milk) is indicated by curd formation and change in the color of the pH indicator. Samples contain residues (Positive samples) showed no clotting or color change (Mohsenzadeh & Bahrainipour, 2008; AL-Dabbagh, 2012).

Disc Diffusion Assay

Using a sterile forceps, commercially available blank discs were immersed into the milk sample until saturation, then the disc was placed on the agar surface of previously prepared agar plates containing spores of *B. subtilis*. The plates were then inverted and incubated at 37-38 °C for 24 hours, till a visible growth was attained. Presence of antibiotic residues in milk samples was indicated by the appearance of inhibition zone around the disc, while the absence of antibiotic residues was indicated by a normal bacterial growth (Salman *et al.*, 2013; Almanzool & Ahmed, 2016).

Acidification method

This method is one of commonly used screening tests of milk samples and was carried out as descried previously (Titouche *et al.*, 2013). It is based on mixing certain volumes of bacterial endospores, pH indicator, and a nutrient agar with the milk sample. The challenged endospore belongs to *Bacillus stearothermophillus* (Academic Research and Activity Center (ARAC), Knowledge University, Erbil, Irak), while the pH indicator is bromocresol purple (HiMedia, India). A colour change of the pH indicator from purple to yellow occurs in response to bacterial growth and production of acidic by-products. When milk sample contains antibiotic residues, the growth of the *B. stearothermophillus* is halted; hence, the colour of the pH indicator remains purple.

Statistical analysis

Data were analyzed using the SPSS software version 25. Confidence intervals of occurrence were estimated using "exact" Clopper-Pearson method at alpha level of 0.05. Chi square test was used to evaluate the differences between groups.

Results

Prevalence of antibiotic residues

Results of the three assays are summarized separately below. The yoghurt culture test detected antibiotic remnants in more samples than the other tests. However, no significant different between the three tests was found ($P = 0.384$). A worth mentioning point is that in all tests, sale points showed more contaminated samples.

Yoghurt culture and acidification assays

Out of 450 raw sheep milk samples, up to 15.8% were positive for the presence of antibiotic residues detected by YCT and 14.4% were positive by disc diffusion assay (Table 1). Statistically, 12.70-19.43% of sheep raw milk sold in Erbil is expected to contain antibiotic residues. There is no significant difference between the two assays in terms of residues detection capacity ($\chi^2 = 0.366$, $P = 0.545$).

Disc diffusion assay

The agar-based assay revealed a slightly lower occurrence of antibiotic residues than the other two tests. Of note, the percentage of positive samples among sale points milk (16.4%) was slightly higher than the level found in farm samples (12.6%) (Table 2).

The change in occurrence rate of antibiotic residues was observed throughout study period (Figure 1**)**. Based on yoghurt culture and disc diffusion tests, the highest frequency of antibiotic residues was noticed in October and November, while the lowest rate was found in August (9.3%). There is a minor association ($r^2 = 0.481$) between progress of summer-autumn months and increase in occurrence of antibiotic residues in milk.

Table 1. Occurrence of antibiotic residues in sheep raw milk samples according to yoghurt culture and acidification tests

Collection site	No. examined	Positive samples n (%)	95% CI	P value
Yoghurt culture test				
Farms	230	32 (13.9)	9.72 – 19.71	0.269
Sale points	220	39 (17.7)	12.92 – 23.43	
Total	450	71 (15.8)	12.70 – 19.43	
Acidification test				
Farms	230	35 (15.2)	10.83 – 29.52	0.228
Sale points	220	43 (19.5)	14.52 – 25.41	
Total	450	78 (17.3)	13.95 – 21.15	

Discussion

The presence of antimicrobial residues in milk and dairy products is considered a serious threat to public health. Consequently, simple and efficient screening tests are important for the determination of residues of different classes of antimicrobials in food and particularly in milk (Kirchhelle, 2018; Islam *et al.*, 2020). In the current work, the occurrence rate of antibiotic residues in sheep milk samples was (15.8%) and (14.4%) according to yoghurt culture and disc diffusion assays. The highest rates (17.7% and 16.4%) were found in milk samples collected from sale points, while the lowest (13.9% and 12.6%) rates were documented in farm milk consequently (Table 1). These findings are consistent with a Jordanian study also used yoghurt culture test and found up to 15.0% of the samples to contain antibiotic residues inferred by clotting just after 2.5 hours of incubation (AL-Kurdi, 2011). Additionally, a similar study from Kenya documented antibiotic residues in 15.5% samples from farmers and 18.4% of samples from vendors (Ondieki *et al.*, 2017).

A slightly lower rates were also reported from Ethiopia (12%) (Abebew *et al.*, 2014), Punjab (9.2-12.9%) (Gaurav, 2014), and Kosovo (10%) (Muji *et al.*, 2018). Still other studies have reported much lower proportions as low as 5% (Kumarswamy *et al.*, 2018; Mollaei *et al.*, 2018; Mottaghiyanpour *et al.*, 2018). However, higher levels of residues were also reported from different countries. For instance, 24% to 34% of samples were positive in different Iranian studies (Aalipour *et al.*,

Table 2. Occurrence of antibiotic residues in sheep raw milk samples according to agar disc diffusion assay

Collection site	No. examined	Positive samples N (%)	95% CI	P value
Farms	230	29 (12.6)	8.61 – 17.60	0.254
Sale points	220	36 (16.4)	11.73 – 21.93	
Total	450	65 (14.4)	11.50 – 17.99	

Temporal variations of antibiotic residues in sheep milk

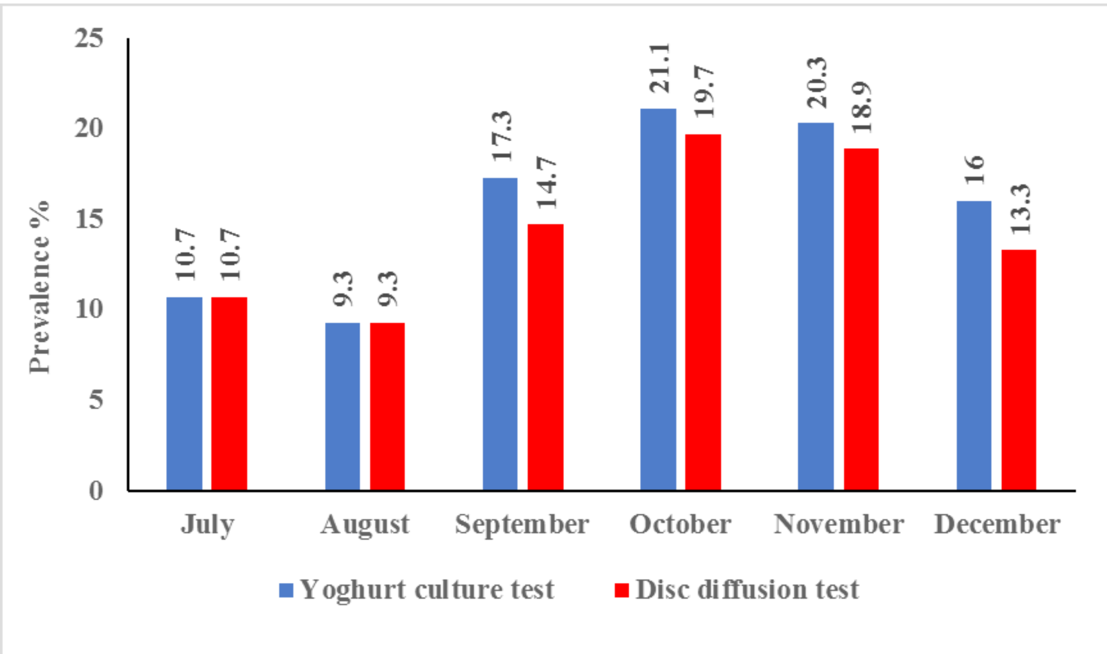

Figure 1. Temporal variations of antibiotic residues occurrence in sheep raw milk samples

2013; Moghadam *et al.*, 2016). In a nearby Iraqi city (Mosul) ewes raw milk samples showed a positive result of 33.3% (AL-Dabbagh, 2012) and much higher prevalence was reported recently by Nigerian scholars (40% to 76%) (Olatoye *et al.*, 2016; Stella *et al.*, 2020).

The observation that milk samples from sales points are more contaminated with residues than farms samples is most likely attributed to the multiple sources of milk received by the sale points. Indeed, there may be farms with higher contamination levels were not sampled in this study. Variations between farms within the same district is also well-known (Founou *et al.*, 2018; Gonzalo *et al.*, 2010; Oliver *et al.*, 2020; Yusuf *et al.*, 2017). Food manufactures should test milk for antibiotic at several points in the supply chain, including farms along with collection centers/points and outlets (FSA, 2015).

Regarding the temporal variations of residues percentage in milk samples, Grădinaru and collaborators found that by early summer there was a decrease in the number of polluted samples, and during the last months of summer and early autumn there was an additional increase in the number of impure samples (Grădinaru *et al.*, 2011). These findings are in good agreement with the results obtained in the present study (Figure 1). Additionally, other similar findings were also reported from Iran, where the wet season of the year, along with increase in weather temperature, the occurrence of polluted samples was raised (Movassagh, 2012). It is perhaps due to the higher frequencies of diarrheal diseases in the food producing animals, which result in the augmentation of antibiotic administration to these animals.

Several control strategies to lower antibiotic residues in milk are well-known. Standard veterinary care and regular health evaluation of livestock animals are important factor. Early detection of disease and administration of appropriate antibiotic for the proper period is an effective practice to ensure better outcomes especially when withdrawal periods are followed as recommended. Workers at sale points should be educated regarding the proper screening and handling of milk received from farms located at distant locales (Jones, 2009; Sachi *et al.*, 2019).

Conclusions

Occurrence of antimicrobial residue in milk are one of an important public health challenge for community, and is one of the most significant milk-borne hygienic issues worldwide. The frequency rate of antibiotic residues among raw sheep milk samples collected from Erbil governorate is high. Regarding the temporal variations between antibiotic residues occurrence and period of study, a minor association between increase in antibiotic residues prevalence and progress of summer-autumn months. It is crucial to promotion awareness of the society concerning hazards of antibiotic residues among milk on public health. Further studies inspecting the impact of heat processing particularly pasteurization process on the stability of antibiotic residues among milk is also recommended.

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