Screening of antibiotic residues in raw milk of cows and buffalos by diffusion assays

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Abstract

Although antibiotics are valuable drugs for treatment of certain infections, their presence in foodstuff derived from animals is a potential public health hazard. They pose a serious threat as they are implicated in direct toxicity; allergic reactions; disturbance of the normal gut microbiota, carcinogenesis, and emergence of antibiotic-resistant bacteria. This study investigated the occurrence of antibiotic residues in raw milk samples derived from cows and buffaloes. Samples were collected randomly from different retail outlets in Erbil city (Iraq) from January 1st to June 30th, 2019. The residues were detected by two diffusion assays against *Bacillus subtilis* bacteria on agar plates. The total occurrence of residues ranged from 11.9% to 13.4% of screened milk samples. No significant differences were found between milk type or location of animal rearing (urban or suburban). Regarding the seasonal variations, spring was found to be associated with gradual decrease in antibiotic residues levels in milk. Such occurrence rate of residues is alarming and require authorities to observe the quality of raw milk introduced to markets for consumption. Further evaluation of antibiotic stability period in raw milk is also necessary.

Introduction

Since the early discovery of antibiotics in late 1920s, these drugs play important roles in various fields including agriculture, food industry, veterinary and human medicine (Finch *et al*., 2010; Meek *et al*., 2015). In dairy farms, antibiotics have been used for various purposes including treatment or prevention of numerous infectious diseases, increase milk production, increase feed efficiency, growth promotion, improving digestion, weight gain, and rise feed conversion ratio (FCR) (Bacanlı and Başaran, 2019; Ezenduka *et al*., 2019; Van Boeckel *et al*., 2015).

Generally, administered antibiotics are eliminated from animal body mostly in urine, and to a lesser extent, through feces. Nonetheless, residues of antibiotics may remain in animal-derived food such as milk, meat, and eggs (Beyene, 2016; Stella *et al*., 2020). The European Union and the American Food and Drug Administration (FDA) define the antibiotic residues as "pharmacologically active substances (whether active principles, recipients or degradation products) and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medical products in question have been administered" (Sachi *et al*., 2019).

Presence of antibiotic residues in milk is considered a violation of food safety standards. Such violations may raise from poor awareness among farmers and/or inadequate literatures supplied by manufacturers of antibiotic products of veterinary uses (FSA, 2015; WHO, 2017). Intentional rush to increase sells may also contribute to the problem. To resolve this problem, farmers should adhere strictly to withdrawal periods before producing foodstuffs from treated animals (Jayalakshmi *et al*., 2017; Rossi *et al*., 2018). Withdrawal period is defined as the time required after administration of a drug to cattle needed to assure that antibiotic residues are below the maximum residue limit_in the marketable milk or other animal-derived foodstuff (Sachi *et al*., 2019; Xu *et al*., 2015).

Consumption of food contaminated with antibiotic residues is associated with health risks including direct toxicity, disturbance of the normal GIT microbiota, bone marrow dysfunctions, congenital anomalies, carcinogenesis, mutagenic effect, and allergic reactions ranging from mild skin rashes to life-threatening anaphylaxis. Additionally, emergence of antibiotic-resistant bacteria was also linked to exposure to sub-lethal concentrations of antibiotics (Sachi *et al*., 2019). Moreover, milk industry is also affected by antibiotic residues since fermentation starter bacteria may also be inhibited by the remnants of the antibiotics (Kebede *et al*., 2014; Maharjan *et al*., 2020).

Milk is a widely consumed food owing to its nutritional value. In Erbil governorate, antibiotic residues in meat of different animals, including milk-producing ones, were evaluated, but their milk is still unaddressed (Al-Mashhadany, 2019, 2018). Therefore, the objectives of this study were to detect antibiotic residues in raw milk of cows and buffaloes sold at retail outlets. The association between months and detection of antibiotic residues among raw milk samples was also investigated.

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Materials and methods

Study design and sampling

During the period from January 1st to June 30th 2019, a total of 320 raw milk samples (170 from cows and120 from buffaloes) were randomly collected from retail milk shops in different markets in Erbil governorate according to previously published method (Almashhadany and Osman, 2019). The collected samples were transported to Department of Medical Lab Science (DMLS), at Knowledge University under chilling condition.

Preparation of the spore suspension

Spores' suspension of *Bacillus subtilis* was prepared according to a standard method (Al-Mashhadany *et al*., 2018). Briefly, heavy inoculums of *B. subtilis* were introduced to the surface of a Nutrient agar plate (HiMedia, India) and incubated at 30°C for 10 days to induce sporulation. After the incubation period, growth was harvested into 10 mL of sterile normal saline and heated at 70°C for 10 minutes to kill the vegetative cells. The heated suspension was centrifuged at 3000 rpm for 10 minutes and the clear supernatant was discarded. Another 10 mL of sterile saline were added to wash off debris of vegetative cells. The mixture was concentrated at the same

speed and duration. The process was repeated twice to obtain a pure suspension of endospore and visually matched to the turbidity of McFarland's 0.5 standard solution equivalent to ≈1.5×10^8 CFU/mL.

Preparation of test plates

Muller-Hinton (MH) agar was prepared as recommended by the manufacturing company (HiMedia, India). After cooling to approximately 45°C, an inoculum of 0.1 mL of spore suspension was introduced to each 100 mL of the agar before solidification. The molten agar was poured into Petri dishes and allowed to solidify at room temperature. Plates were used at the same day of preparation or held at refrigerator and used within one week.

Detection of antibiotic residues

Well diffusion assay

Well diffusion assay was followed to determine the presence of antibiotic residues in raw milk samples. Five wells (7 mm diameter) were cut in MH agar using sterile cork borer separated by 20 mm apart from one another. A volume of 0.1 mL of milk samples was added to each of the 5 wells. The Petri plates were incubated for 24 h at 36±1ºC, under aerobic conditions. Presence of antibiotic residues was inferred by formation of inhibition zone around the wells (absence of bacterial growth), while the absence of antibiotic residues in the sample was indicated by an evenly distributed bacterial growth including around the wells (Al-mohana *et al*.,2010; Valgas *et al*., 2007).

Disk diffusion assay

Blank disks (12 mm) of filter papers (Whatman 1) were totally soaked into pre-prepared sample and placed on the surface of agar medium containing *B. subtilus* using sterile forceps. In addition to the test disk, a control blank disk was also added in every agar plate. The Petri plates were incubated at 36±1ºC for 24 hrs. The presence of antibiotic residues (positive results) was indicated by formation of transparent zone around the disk, as in well diffusion method (Kumarswamy *et al*., 2018; Popelka *et al*., 2004; Salman *et al*., 2013).

Statistical analysis

Data were analyzed by the SPSS software version 25. Confidence intervals of prevalence were estimated using Clopper-Pearson method at alpha level of 0.05. Chi square test was used to test the difference between groups.

Results

Occurrence of antibiotic residues in raw milk

Out of 320 raw milk samples, 13.4% were positive for the presence of antibiotic residues detected by agar well diffusion assay and 11.9% of samples were positive by the disk diffusion (Table 1). The proportion of positive samples among cow milk samples was 12.9% which is higher than the proportion found in buffaloes' milk (10.7%) (Table 2). However, no significant differences were detected between raw milk type (Table 1) or the assay methods employed for the screening of samples (p=0.568). Statistically, it is estimated that 9.90% to 17.67% of raw milk obtained from cows and buffaloes in Erbil might contain antibiotic residues at detectable level by agar well diffusion.

Incidence of antibiotic residues according to sampling location

Regarding to the distribution of antibiotic residues in examined samples, the results indicated occurrence rates of 12.7% (19/150) and 14.1 % (24/170) of cow and buffaloes milk from urban and suburban area respectively (Table 2). There is no significant difference between locations in terms of antibiotic residues level (χ^2=0.134, p=0.7145).

Temporal variations of antibiotic residues during study period

The change in occurrence of antibiotic residues was observed throughout study period (Figure 1). According to well diffusion assay, the highest frequency of antibiotic residues was noticed in February (24.1%) and March (20.8%), while the lowest rate was found in June (5.7%). On the other hand, the highest occurrence of residues was observed in February (20.4 %) and March (18.9%), while the lowest rate was found in June (5.7%) according to the disk diffusion assay (Figure 1). Collectively, there was a strong association (r^2=0.935 & r^2=0.847) between decrease of residues and the period from late winter to late spring (February to July).

Discussion

Early reports of antibiotics residue in milk were first published in 1960s, followed by a surge increase in detection after 2000s. Such issue received much attention in recent years because of growing food safety and public health worries (Molina *et al*., 2003; Sachi *et al*., 2019). Their occurrence

Table 1. Occurrence of antibiotic residues among cow and buffaloes milk samples.

Milk source	No. examined	Positive samples n (%)	95% CI	P value
Well diffusion assay				0.480
Cows	170	25 (14.7)	9.75-20.94	
Buffaloes	150	18 (12.0)	7.27-18.30	
Total	320	43 (13.4)	9.90-17.67	
Disk diffusion assay				0.544
Cows	170	22 (12.9)	8.29-18.94	
Buffaloes	150	16 (10.7)	6.22-16.74	
Total	320	38 (11.9)	8.54-15.93	

Table 2. Occurrence of antibiotic residues in urban and suburban areas according to well diffusion assay.

Milk source	Urban area*		Suburban areas	
	No examined	Positive n (%)	No. examined	Positive n (%)
Cows	80	11 (13.8)	90	14 (15.6)
Buffaloes	70	8 (11.4)	80	10 (12.5)
Total	150	19 (12.7)	170	24 (14.1)

*According to Urban Planning Authority, urban area is the main town, while locations adjacent to the city are the suburban areas.

of antibiotic residues in food of animal origin (Okocha *et al*., 2018; Savarino *et al*., 2020).

In the present study, the frequency of milk samples contaminated with antibiotic residues was 13.4% and 11.9% according to well diffusion assay and disk diffusion assay, respectively (Table 1). These findings are consistent with various studies from Kenya, Kosovo, and Iraq with a total occurrence range from 10% to 18.4% (Kang'ethe *et al*., 2005; Ondieki *et al*., 2017; Muji *et al*., 2018; Al-mohana *et al*., 2010). Nonetheless, lower occurrence rates were reported from Montenegro, Kosovo, and India where the occurrence ranged from 6.0% to 8.48% (Kumarswamy *et al*., 2018; Nikolić *et al*., 2011; Rama *et al*., 2017). On the other hand, higher rates have been reported recently from Kenya, (15.5% - 18.4%) (Ondieki *et al*., 2017), Iran (34%), and Nigeria (40.8% to 76%) (Olatoye *et al*., 2016; Stella *et al*., 2020). Such variations might have resulted from different factors such as the degree of adherence to withdrawal period, extent of arbitrary use of antibiotics, stability period of the antibiotics in milk, and detection methodology (Sachi *et al*., 2019).

High rates of antibiotic residues were associated with milk from suburban or rural farms in Kenya and Nigeria (Kang'ethe *et al*., 2005; Orwa *et al*., 2017; Yusuf *et al*., 2017). These findings are in good agreement with the results obtained in the present work (Table 2), where the occurrence in urban area was (12.7%), while in suburban area was (14.1%). In non-urban areas, farmers' awareness and safety standards evaluation of raw milk are most likely inadequate or absent, which may explain the increase of antibiotic residues in milk obtained from such locales. It is obvious that there is an association between decrease in occurrence of antibiotic residues and winter-spring seasons progress (Figure 1). Occurrence of antibiotic residues in spring was also found to be significantly lower than winter (wet season) in a recent Iranian study (Moghadam *et al*., 2016). In Erbil governorate, winter is associated with an average rainfall of 56-80 mm. Nonetheless, the seasonality of antibiotic residues levels seems multifactorial and complicated. Bacterial infections, especially diarrheal and respiratory diseases, in cattle were found to predominate during the wet season (Parvez *et al*., 2014). Such infections result in the augmentation of antibiotic administration antibiotics more than other seasons which may explain the gradual decrease of residues as winter progress to spring. However, contradictory findings were reported from Kenya, Iran, and Romania (Gradinaru *et al*., 2011; Kang'ethe *et al*., 2005; Movassagh, 2012; Najim and Al-kurashi, 2017). Still other researchers found no association between seasons and level of antibiotic residues (Aalipour *et al*., 2013).

There are several strategies to avoid antibiotic residues in milk. Continuous examination and medical assessment of cattle to avoid spread of infections are the most important. Once infection is detected, appropriate administration of antibiotics and strict adherence to withdrawal period is a necessary practice. Farmers' awareness is an important factor to reduce the contamination level by antibiotic residues (Jones, 2009; Sachi *et al*., 2019).

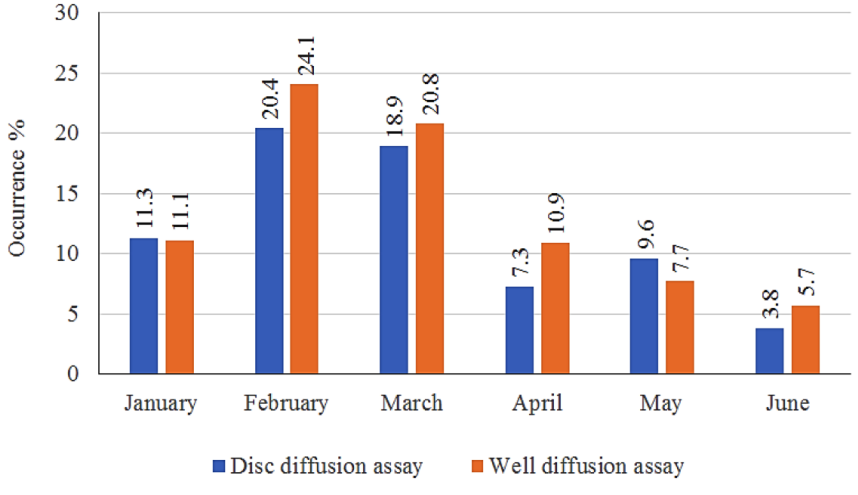

Figure 1. Variations of antibiotics residues in raw milk at time scale during study period.

Conclusions

Presence of antibiotics residues in cow and buffaloes milk is one of global public health challenges for raw milk quality. According to our results, the occurrence of antibiotic residues in raw milk samples (cows and buffaloes milk) collected from Erbil governorate is generally high with no significant differences between samples originated from urban and suburban locations with a gradual decrease as spring progress. Promotion of farmers' awareness about hazards of antibiotic residues in milk is a pivotal strategy to mitigate the negative consequences of antibiotic remnants in milk. Further investigations of heat impact especially pasteurization process on the stability of antibiotic residues in milk are recommended. National and international preventive strategies should be applied to protect consumers from health hazards that result from consumption of milk contaminated with antibiotics residues.

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