Original Article

Evaluation of Antibiotic Residues in Raw Milk at Erbil Governorate, Iraq: Using Four Detection Methods

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ABSTRACT

Background: Massive animal production has been aided by medicinal products, especially antibiotics. Unfortunately, remnants of such drugs remain in animals' tissues or are eliminated in their milk or eggs.

Objectives: This study aimed to highlight the hazards of antibiotic residues in milk and to assess the frequency rate of these residues in the milk of cows, sheep, and goats.

Methods: One hundred fifty samples (50 for each cow, sheep, and goats) were collected randomly from different farms and retail outlets in Erbil Governorate, from April 1 to June 30, 2024. The residues were detected using disc diffusion assay (DDA), well diffusion assay (WDA), Delvotest, and enzyme-linked immunosorbent assay (ELISA).

Results: The frequency of residues according to DDA, WDA, and Delvotest were as follow: 12%, 14%, 14% in cow milk; 10%, 14%, 14% in sheep milk; and 10%, 12%, 16% in goat milk, respectively. ELISA results showed that 29 samples of cow milk, 20 sheep milk, and 30 goat milk samples contained beta-lactam antibiotics. The antibiotic residues in milk samples from urban areas were higher (11.6% and 14.5%) than those from rural areas (9.9% and 12.3%). Temporal variations showed the highest antibiotic residue level in April (20.9%) and the lowest (8.9%) in June.

Conclusion: The frequency rate of antibiotic residues in cow, sheep, and goat milk samples collected from Erbil Governorate is high. It is crucial to promote awareness in the society concerning hazards of antibiotic residues in milk on public health.

Keywords: Antibiotic residues, Delvotest, ELISA, Milk, Public health

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Introduction

In the last decades, massive animal production has been aided by medicinal products, especially anti-infective drugs (Manyi-Loh et al., 2018). Antibiotics are usually administered to milk-producing animals in drinking water or feed. Sub-therapeutic doses are commonly used as a prophylaxis to healthy animals. Nearly all the antimicrobials used for humans are also used for food-producing animals, including the most modern classes of antibiotics. The global use of antibiotics in livestock is 63.151±1.56 tons/year (Van Boeckel et al.,2015). Unfortunately, remnants of such drugs remain in animals' tissues or are eliminated in their milk or eggs (Al-Shuwaili & Khudhir, 2022). Regulatory authorities affiliated with the European Union (EU) and Food and Drug Administration (FDA) have defined the antimicrobial residues (AMRs) as substances associated with impurities of pharmacologically active compounds in foodstuffs derived from treated animals (Almashhadany et al., 2022). Based on this criterion, even well-controlled administration of antimicrobial drugs will leave AMRs in animal tissues or products for a specific period, called the withdrawal period (WDP) (Bacanlı & Başaran, 2019).

Additionally, failure to follow antibiotic manufacturers' instructions also accounts for the occurrence of residues in milk. In veterinary medicine, the WDP of a drug is defined as the minimum time between the administration of the last dose and the production of meat or milk from the treated animal after which the AMRs are lower than the maximum residue limit (MRL) (Abo El-Makarem et al., 2020). WDP varies widely (ranges from hours to weeks) between different drugs, as it depends on the chemical nature of the drug, the dose, the route of administration, and the species of the animal (Almashhadany, 2019). Most public health authorities consider the presence of AMRs as an illegal practice in animal-derived foods above the MRL. In turn, the MRL is defined by regulatory authorities as the highest concentration of a compound in food materials that is safe for animal or human consumption at a specific time. The presence of AMRs in milk and dairy products have been a major concern for public health officials. Quality control systems require accurate and cost-effective quantification approaches to detect AMRs in milk and dairy products (Almashhadany, 2021). Additionally, administration at minimum doses of antimicrobials, usually as a food additive, may be practiced to suppress gut bacteria, leaving more nutrients for animals to be absorbed.

Irrational use of antimicrobial drugs during treatment of infections is the main source of AMRs in milk (Zhang et al., 2009). Furthermore, indiscriminate addition of antimicrobial agents as food additives is another important source. Contamination of milk starts right from the intake of contaminated pasture or/and water by the animal (Arif, et al., 2021). The contaminants may also be introduced, with lesser extent, during milk collection, preservation, transport, processing, and packaging (Shaikh & Patil, 2020).

Ideally, milk should not contain any constituents or any exogenous chemicals that may result in toxicity or other health issues in consumers. Not only are direct consumers affected by AMRs, but also the starter cultures in the dairy industry are affected negatively, leading to economic losses (Beyene et al., 2015). The gut microbiota varies from person to person, but it plays similar roles in competing with pathogens and providing vitamins. Disruption of a balanced microbial population in the gut can trigger diseases of different nature at different ages, ranging from allergies at an early age to inflammatory bowel disease (IBD) in young adults. AMRs from broad-spectrum antibiotics may inhibit a wide range of gut microbiota and provide an opportunity for pathogenic species to expand their populations and initiate diseases.

The development of antimicrobial resistance by pathogens has been a global concern since the early 1960s. Misuse of antibiotics has been the main contributing factor for the emergence of resistant strains that were naturally susceptible to different antibiotics. A recent alarming report estimated that up to 10 million individuals would be victims of drug resistant pathogens by 2050 unless the problem of resistance is mitigated (de Kraker et al., 2016).

Consumption of milk or dairy products containing residues of tetracycline or azithromycin has been found to cause lifelong discoloration of the nails and teeth in children and to impair the immune and cardiovascular systems (Kurjogi et al., 2019).

Milk is an extensively consumed nutritious food owing to its highly nutritional value. There was inadequate information on the levels of AMRs in milk in the Erbil Governorate. Hence, the aim of this study was to assess the frequency of AMRs in the milk of cows, sheep, and goats in that area. The relationship between months and the detection of AMRs among cow, sheep, and goat milk was also investigated.

To detect ARs, an agar disk-diffusion assay developed in 1940 is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. Nowadays, many accepted and approved standards are published by the Clinical and Laboratory Standards Institute (CLSI) (Clinical Laboratory Standards Institute, 2018).

Materials and Methods

Study design and milk sampling

This study was conducted from April to June 2024. One hundred and fifty milk samples were collected from lactating cattle, sheep, and goats (50 samples for each) from different farms and retail markets of rural and urban areas in Erbil governorate, Iraq. For each sample, about 200 mL of milk sample was collected into labelled sterile plastic containers with screw lids under hygienic conditions. On the same day of collection, all the samples were transported under cool conditions (inside an icebox at ~ 5 ˚C) to the Department of Medical Laboratory Sciences at the College of Science, Knowledge University, Erbil, Iraq. In the laboratory, each sample was divided into four parts and stored in a deep freezer at −18 ˚C until further analysis.

Detection of antibiotic residues using disk diffusion assay (DDA)

Using sterile forceps, commercially available blank discs were immersed into the milk sample until saturated, and then the disc was placed on the agar surface *Bacillus subtilis*. The plates were then incubated at 37–38 ˚C for 24 hours until visible growth was attained. The presence of antibiotic residues in milk samples was indicated by the appearance of an inhibition zone around the disc, whereas the absence of antibiotic residues was indicated by normal bacterial growth (Almashhadany, 2021).

Detection of antibiotic residues using WDA

A well diffusion assay was followed to determine the presence of antibiotic residues in raw milk samples. Five wells (7 mm diameter) were cut in Mueller–Hinton agar using sterile cork borer, at 20 mm intervals 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±1 ˚C under aerobic conditions. The presence of antibiotic residues was inferred by the formation of an 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.

Detection of antibiotic residues using Delvotest

The method was carried out according to the instructions by the manufacturer (DSM food specialties B.V., Delft City, The Netherlands). Thus, 0.1 mL milk samples were added to individual Delvotest cups preloaded with *Bacillus stearothermophilus* var. calidolactis and a pH indicator in a compact medium, and then the cups were incubated at 64 ˚C for 195 minutes. Results were distinguished visually by interpreting the color change of the pH indicator. Fail (negative): if the color does not change (remains purple) the bacteria have not developed due to the presence of antibiotic residues. Pass (positive): if the color changes (yellow), the bacteria have developed, indicating no antibiotic residues in the sample (Layada et al., 2016).

Detection of antibiotic residues using enzyme-linked immunosorbent assay (ELISA)

All samples showing inhibition zones wider than 2 mm on at least one plate with *B. subtilis* were examined further for Beta-lactams with a commercially available ELISA kit (Shanghai Ideal Medical Technology Co., Shanghai, China). First, 50 µL of standard solution were added to 6 standard wells. Then, 10 µL of each sample was added to 40 µL of diluent; this step did not add anything to the blank wells at this stage. After that, 100 µL of HRP-conjugate reagent were added to each well, and the wells were covered with an adhesive seal and incubated for 60 minutes at 37 ˚C. The wells were then aspirated and washed; the process was repeated four times for a total of five washes. Chromogen solution A (50 µL) and chromogen B (50 µL) were added to each well, gently mixed, and incubated for 15 minutes at 37 ˚C in darkness. Finally, 50 µL of stop solution was added to each well. The color changed from blue to yellow. Optical density was read at 450 nm using a microtiter plate reader within 15 minutes (Gaurav et al., 2014).

Results

Occurrence of antibiotic residues in cow milk samples

According to DDA test results, the prevalence of antibiotic residues in samples from farm was 11.5%, while in samples from retail outlets it was 12.5% (Table 1). According to WDA test results, the prevalence of antibiotic residues in samples from farm was 11.5%, while in samples from retail outlets it was 16.7% (Table 2). According to Delvotest results, the prevalence of antibiotic residues in samples from farm was 19.2%, while in samples from retail outlets it was 12.5% (Table 3).

Table 1. Presence of antibiotic residues in milk samples from different sources based on the DDA

Animal	Source of Milk	No. Examined	No. (%) Positive
Cow	Farms	26	3(11.5)
	Retail outlets	24	3(12.5)
	Total	50	6(12)
Sheep	Farms	28	3(10.7)
	Retail outlets	22	2(9.1)
	Total	50	5(10)
Goat	Farms	27	2(7.4)
	Retail outlets	23	3(13.1)
	Total	50	5(10)

Frequency of antibiotic residues in sheep milk samples

According to DDA test results, the frequency of antibiotic residues in sheep milk samples from farm (10.7%) was higher than that in samples from retail outlets (9.1) (Table 1). According to WDA test results, the frequency of antibiotic residues in sheep milk samples from farm (14.3%) was somewhat higher than that in samples from retail outlets (13.7) (Table 2). According to Delvotest results, there were 14.3% positive samples from farms and 13.7% positive samples from retail outlets (Table 3).

Detection of antibiotic residues in goat milk samples

According to DDA test results, the prevalence of antibiotic residues in samples from farm was 7.4%, while in samples from retail outlets it was 13.1% (Table 1). According to WDA test results, the prevalence of antibiotic residues in samples from farm was 11.1%, while in samples from retail outlets it was 13.1% (Table 2). According to Delvotest results, the prevalence of antibiotic residues in samples from farm was 14.8%, while in samples from retail outlets it was 17.4% (Table 3).

Table 2. Presence of antibiotic residues in milk samples from different sources based on the WDA

Animal	Source of Milk	No. Examined	No. (%) Positive
Cow	Farms	26	3(11.5)
	Retail outlets	24	4(16.7)
	Total	50	7(14)
Sheep	Farms	28	4(14.3
	Retail outlets	22	3(13.6)
	Total	50	7(14)
Goat	Farms	27	3(11.1)
	Retail outlets	23	3(13.1)
	Total	50	6(12)

Table 3. Presence of antibiotic residues in milk samples from different sources based on the Delvotest

Animal	Source of milk	No.	No. (%)
		Examined	Positive
Cow	Farms	26	5(19.2)
	Retail outlets	24	3(12.5)
	Total	50	8(16)
Sheep	Farms	28	4(14.3)
	Retail outlets	22	3(13.7)
	Total	50	7(14)
Goat	Farms	27	4(14.8)
	Retail outlets	23	4(17.4)
	Total	50	8(16)

Result of antibiotic residues in all milk samples rural and urban areas

The rate of antibiotic residues detection, according to DDA, WDA, and Delvotest, was 10.7%, 13.3%, and 15.3%, respectively. Higher rates of antibiotic residues detection according to Delvotest were associated with milk from rural areas (16.1%) compared with urban areas (14.1%) (Table 4).

Detection of beta-lactam antibiotics in all milk samples according to ELISA

ELISA analysis results showed that 29 samples of cow milk, 20 sheep milk, and 30 goat milk contain beta-lactam antibiotics (Table 5).

Temporal variation of antibiotic residues in all milk samples according to WDA

Regarding the temporal variations in antibiotic residues occurrence, there was a minor association between an increase in antibiotic residues prevalence and the progression of spring to summer months. However, the frequency of antibiotic residues during April, May, and June was 20.9%, 11.8%, and 8.9%, respectively (Figure 1).

Discussion

Antibiotics have long been used for prophylactic purposes and for the prevention and treatment of infectious diseases in dairy animals. However, in recent decades, this has become a matter of concern due to the widespread belief that there has been misuse or abuse of these drugs in food-producing animals such as cattle, sheep, and goats. This misuse has led to the occurrence of residues in their milk and dairy products. Early reports on the presence of antibiotics residues in milk were first published in 1960s, followed by a surge in detection after 2000s (Sachi et al., 2019). Such an issue has received much attention in recent years because of growing food safety standards and public health concerns. Therefore, this study was conducted to evaluate the levels of antibiotic residues in raw milk in Erbil Governorate.

Table 4. Comparison of different screening methods for detecting antibiotic residues

Source of milk	No. of Samples	No. (%)		
		Positive Samples (DDA)	Positive Samples (WDA)	Positive Samples (Delvotest)
Rural	81	8(9.9)	10(12.3)	13(16.1)
Urban	69	8(11.6)	10(14.5)	10(14.1)
Total	150	16(10.7)	20(13.3)	23(15.3)

DDA: Disc diffusion assay; WDA: Well diffusion assay.

Table 5. Beta-lactam levels in different milk sources

Parameters	Mean±SEM		
	Sheep (20)	Cow (29)	Goat (30)
Antibiotic residues (Unit/mL)	2.510±0.098[a]	2.907±0.056[b]	3.184±0.061[c]

Note: Different letters indicate a significant difference at P<0.05.

Occurrence of antibiotic residues in cow milk

According to DDA results, out of 50 raw milk samples, the prevalence of antibiotic residues in samples from farms was 11.5%, while in samples from retail outlets it was 12.5%. The results of this study are in agreement with a study conducted by Almashhadany (2021b), who reported 11.9% contamination with AMRs according to the DDA. These findings are consistent with various studies conducted by Kung et al. (2005) from Kenya, Muji et al. (2018) from Kosovo, and Al-mohana et al. (2010) from Iraq with a total rates range from 10% to 18.4%. However, lower frequency rates were reported from Montenegro, Kosovo, and India, where the prevalence ranged from 6% 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%) (Nemati Niko et al., 2020), 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 WDPs, the extent of arbitrary use of antibiotics, the stability period of the antibiotics in milk, and the detection methodology (Sachi et al., 2019).

The detection rate of antibiotic residues in cow milk according to WDA was 14.0%. Our result is in agreement with the rate mentioned by Almashhadany (2021) who reported that the detection rate of antibiotic residues in cow milk was 13.4% according to WDA. The occurrence of antibiotic residues in cow milk according to Delvotest showed a frequency rate of 16.0%, which is lower than the 33% reported by Mohammed & Salman (2020).

Frequency of antibiotic residues in sheep milk

Regarding the detection of antibiotic residues in sheep milk, the frequency according to DDA was 10.0%, which is slightly lower than the result reported by Almashhadany (2020), who stated that from 450 sheep milk samples, 14.4% contained AMRs according the DDA. This difference may be due to the size of the studied populations. According to the WDA results, the frequency of antibiotic residues in sheep milk samples from farms was higher than that in samples from retail outlets. Also, the frequency in samples from farms was 14.3%, compared with 13.6% in retail outlet samples. A comparable work from Kenya reported antibiotic residues in 15.5% of samples from farms and 18.4% of samples from sellers (Kung et al., 2005). Slightly lower proportions were also reported from Kosovo (10%) (Muji et al., 2018). According to Delvotest results, out of 50 sheep milk samples, there were 14.3% positive samples from

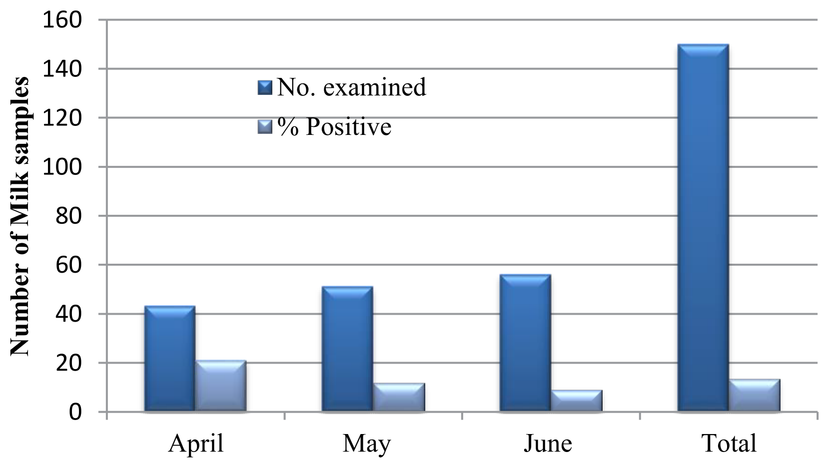

Figure 1. Temporal variation in the frequency rate of antibiotic residues in all milk samples according to the WDA

farms and 13.7% positive samples from retail outlets. In a study by (Fatima et al. (2013) in Algeria, examining antibiotic residues in ewe's raw milk using Delvotest, the residues were found in 4.76% of milk samples.

Detection of antibiotic residues in goat milk

According to DDA results, out of 50 raw milk samples, the prevalence of antibiotic residues in samples from farms was 7.4%, while in samples from retail outlets it was 13.1%. According to Almashhadany (2021) in Iraq, the prevalence rates were 13.5% and 16.4% in farm samples and retail store samples, respectively. No significant differences were found between sampling sites (farms vs sale points). In the study by Ambaw and Aytenfsu, (2021) in Ethiopia, of the 125 milk samples screened, 12% were positive for antibiotic residues.

According to WDA results, the prevalence of antibiotic residues in samples from farms was 11.1%, while in samples from retail outlets it was 13.1%. According to Delvotest results, the prevalence of antibiotic residues in samples from farms was 14.8%, while in samples from retail outlets it was 17.4%. This rate is lower than that reported by Ham et al. (2008); they declared that from 56 milk samples analyzed by Delvotest, 37.5% of samples showed positive results. Ahlberg et al. (2018) analyzed a total of 480 samples with the Delvotest; 24% of samples were positive.

Antibiotic residues in all milk samples from rural and urban areas

This work showed that the detection rates of antibiotic residues according to DDA, WDA, and Delvotest were 10.7%, 13.3%, and 15.3%, respectively. High rates of antibiotic residues according to Delvotest were associated with milk from rural areas (16.1%), with 14.5% from urban areas; this difference was not significant. High rates of antibiotic residues were associated with milk from suburban or rural farms in Kenya. The Charm-AIM screening kit used detected AMRs in up to 16% of marketed milk samples, suggesting an average risk of exposure by consumers of up to five times every month (Kang et al., 2005). In Nigeria, antibiotic residues were obtained in 40.8% fresh milk, 24.4% wara (local cheese), and 62.3% nono (fermented milk), respectively (Orwa et al. 2017).

Detection of antibiotic residues in all milk samples according to ELISA

ELISA analysis results showed that 29 samples of cow milk, 20 samples of sheep milk, and 30 samples of goat milk contained beta-lactam. Gaurav et al. showed that, from a total of 133 cattle milk samples collected from dairies in 5 districts of Punjab State, India, and analyzed by ELISA, 18 samples were found to contain tetracycline residues in the range 16–134.5 ppb (Gaurav et al., 2014). Three samples exceeded the MRLs for tetracycline antibiotics. Hebbal et al. found that, out of the 215 pooled raw milk samples screened for antibiotic residues using the microbial inhibition assay (MIA), 22 samples (10.23%) were positive for antibiotic residues from Palakkad, Kerala, India (Hebbal et al., 2020). Among these 22 positive samples, five (2.33%) were positive for oxytetracycline residues. The mean concentrations were calculated as 201.00±41.25 ng/mL and 272.11±53.21 ng/mL using ELISA and high-performance liquid chromatography (HPLC), respectively. In another recent study using ELISA, it was reported that from 45 milk samples, 48.9% were positive for the presence of antibiotic residues (Nemati Niko et al., 2020). ELISA could be a suitable alternative for the detection of antibodies (Madani et al., 2024).

Temporal variation of antibiotic residues in all milk samples

Regarding temporal variations in the occurrence of antibiotic residues, our results showed a minor association between the decrease in antibiotic-residue prevalence and the progress from April to June. The frequency of antibiotic residues during April, May, and June was 20.9%, 11.8%, and 8.9%, respectively. A recent Iranian study, conducted on 251 samples of commercial pasteurized milk packets distributed in schools (code A), raw milk collection centers in Gonabad City (code B), and pasteurized milk production factories (code C) in Gonabad City, reported that the occurrence of antibiotic residues in spring was also significantly lower than in winter (wet season). From the milk samples most contaminated with antibiotics, 17 samples were positive in January, and for code A, 13 samples were positive in the same month. There was no significant difference among the three types of milk (P>0.05). The highest number of milk samples (n=7) contaminated with antibiotics was related to code B (38.5%). Most positive cases were related to code A in winter. Also, there was no significant difference among the three types of contaminated milk regarding the year and month (P=0.164 and P=0.917, respectively) (Moghadam et al., 2016). Additionally, other similar findings were also reported from Iran: 50 samples of cows' raw milk were collected from April to September 2010 by systematic random sampling. All samples were examined by Copan milk test (CHR. Hansen, Denmark) for the presence of antibiotics residues. Twelve samples (24%) were positive for antibiotic residues. The study revealed that antibiotic residues in milk were high in Bostanabad region (Movassagh, 2012).

The results obtained from different microbiological methods were not identical, but they were close to each other. In the case of the ELISA test, the differences in results in comparison with other methods was significant and high. This test is based on the binding of antibodies and antigens and is technically very accurate. However, it is possible that compounds structurally similar to beta-lactam antibiotics may have increased the number of positive results.

Conclusion

The frequency rate of antibiotic residues in raw milk samples from cow, sheep, and goat collected from Erbil Governorate is high. The results obtained from different methods were not identical, but they were close to each other, except for the ELISA test, which demonstrated significantly higher rates of positive samples. It is crucial to promote public awareness about the hazards of antibiotic residues in milk and to implement monitoring programs.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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