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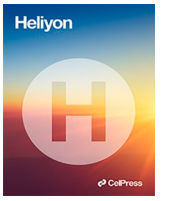

Research article

Residual antibiotics in milk samples: Assessing the risk and prevalence in Bangladesh

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ABSTRACT

The study aimed to analyze five commonly used veterinary antibiotics: tetracycline (TC), oxytetracycline (OTC), doxycycline (DOX), chlortetracycline (CTC), and enrofloxacin (ENR) in different types of milk samples, risk estimation, and to investigate the correlation between the presence of multiple antibiotic residues. About 27 milk samples, such as raw milk from collection centers, processed milk from processing plants, pasteurized, UHT, and flavored milk from retail stores, were examined using RP-HPLC against five veterinary antibiotics in Dhaka, Bangladesh. The correlation between antibiotics was analyzed using Pearson's correlation test. OTC was prevalent in 100 % of the analyzed samples, while CTC, ENR, TC, and DOX were found in 29.63 %, 22.22 %, 18.52 %, and 7.41 % of the samples, respectively. Only OTC residues were detected in both raw and soon-after-processed milk. However, most of the UHT and flavored milk samples showed the occurrence of multiple antibiotic residues. Among the detected samples, 37.04 % exceeded the MRLs, while 44.44 % were near the limit. The mean concentration of TC was higher than others. The correlation test revealed a significant moderate to strong positive correlation between TC, CTC, DOX, and ENR, while OTC showed no significant correlation with the other antibiotics. Risk analysis showed no immediate risk from detected antibiotics but can raise concern due to continuous exposure. The results obtained from this study underscore the importance of adhering to appropriate withdrawal periods and implementing appropriate quality control protocols to ensure the protection of public health.

1. Introduction

Milk and dairy products are widely recognized for their nutritional benefits and are essential to diets across all age groups. As a result, milk consumption has been a significant part of human nutrition for centuries [1]. In 2023, the global milk market is expected to generate US$331.80 billion, with an annual growth rate of 6.0 %. In Bangladesh, this market is anticipated to grow even faster, at 9.5 % annually [2]. This expansion of the dairy industry is largely driven by technological advancements, which have increased milk production and profitability worldwide. Alongside this increase in production, milk consumption has also risen [3]. For this reason, to meet the growing demand for milk and dairy products, antibiotics such as tetracyclines, sulfonamides, macrolides, β-lactams, and

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cephalosporins are used as growth promoters or reproductive cycle enhancers [4] and other group of antibiotics such as fluoroquinolones, aminoglycosides, quinolones, and lincosamides are used for treating diarrhea, mastitis, respiratory diseases, inflammatory diseases, and shipping fever in cattle farming [5]. About 73 % of antibiotics are used to treat disease and promote growth worldwide [6], exceeding the safety level, which ultimately leads to the occurrence of residual antibiotics in animal products [7]. The U.S. Food and Drug Administration (FDA) estimates that around 80 % of antimicrobial agents used in agriculture are intended for animals raised for food production [7]. Furthermore, global sales of antimicrobials are projected to rise by 11.5 % by 2030 [8].

The expanded cattle farming made substantial contributions to the economy of Bangladesh, which led to the indiscriminate usage of antibiotics at the commercial level [9]. As a result, this extensive use of antibiotics resulted in the deposition of its residues or metabolites in various tissues and organs, which are ultimately secreted in milk [7,10], meat [11], fish [12], and eggs [10]. Failure to adhere to proper withdrawal periods, improper handling, monitoring, and screening facilities, and excessive use of antibiotics due to inadequate hygiene and sanitation in cattle can lead to the development of these antibiotic residues in animal products. Exceeding the permissible Maximum Residual Limits (MRLs) of antibiotics in food can cause serious acute health effects such as hypersensitivity reactions and gastrointestinal disorders in humans. Prolonged exposure to these residues can also cause chronic health impacts like alteration of gut microflora, cancer, reproductive effects, leukocytosis, teratogenicity, bone marrow toxicity, and nephropathy [13,14]. Considering the toxicity and potential effects of metabolized antibiotics on human gut microbiota, many regulatory agencies, such as the Food and Agriculture Organization (FAO), the European Union (EU), and the Codex Alimentarius Commission (CAC), have established maximum residue limits (MRLs) and withdrawal periods that vary for each antibiotic [13,15]. Moreover, metabolized antibiotics (40–90 %) are actively eliminated through waste products that pollute the environment and, subsequently, promote the emergence of antibiotic-resistant bacterial strains by exerting evolutionary and selective pressure [7]. This happens because residual antibiotics can undergo chemical regeneration (such as cleavage of acetyl groups), leading to their reactivation and persistence in the environment [16]; for instance, certain antibiotics like tetracyclines, erythromycin, and sulfamethoxazole can remain in the water and soil for over a year [7].

Milk can harbor various microorganisms, including pathogens, which can lead to milk-borne diseases. Common pathogens found in milk include *Campylobacter*, *E. coli* (specifically O157:H7), *Listeria monocytogenes*, *Salmonella*, *Yersinia*, *Mycobacterium tuberculosis*, and several others, with many of them developing resistance [17]. The extensive utilization of antibiotics in congested and unhygienic environments in cattle farming can contribute to the development of antibiotic-resistant pathogens associated with zoonotic diseases, which can also proliferate and spread via the food chains. Consequently, humans face a higher risk of exposure to these antibiotic-resistant zoonotic pathogens if such food is not properly treated [18]. Milk is normally treated through many processes of pasteurization, which makes the milk completely pathogen-free and reduces the total load of microorganisms [19]. However, this conventional heat treatment is insufficient to decrease a significant portion of the antibiotic residues such as amphenicols, lincomycin, and sulfonamides [20], subsequently contaminating the milk with such antibiotic-resistant pathogens. The presence of antibiotic residues (exceeding MRLs) in dairy products, along with the emergence and spread of antibiotic-resistant bacteria, is a significant concern in the public health sector. However, data and literature on antibiotic usage within food-animal production systems, as well as on the subsequent emergence and transmission of antibiotic resistance to humans, remain limited [7].

Various analytical techniques like thin-layer chromatography (TLC), rapid test kits, microbial inhibition test (MIT), enzyme-linked immunosorbent assay (ELISA), Liquid Chromatography (LC) with Mass Spectrometry (MS), UV–VIS spectral analysis, Ultra High-Performance Liquid Chromatography (UHPLC), and High-Performance Liquid Chromatography (HPLC) coupled with fluorescence spectroscopy have been developed to detect and analyze antibiotic residues in dairy products [21]. Moreover, the potential adverse impacts of residual antibiotics on human health can be determined by calculating the risk estimation. The risk estimation procedure for food chemicals, as outlined by the FAO/WHO, comprises four distinct stages: (a) hazard identification, (b) hazard characterization or dose-response assessment, (c) exposure assessment, and (d) risk characterization [22]. The concepts of Hazard Quotient (HQ) and Risk Quotient (RQ) are extensively employed in the analysis of such chemical risk. On one hand, RQ is used to measure environmental threats, while HQ is employed to evaluate health-related risks. This approach is highly regarded for upholding food safety to protect public health [23].

Previous studies conducted in Chittagong and Mymensingh have detected tetracycline, oxytetracycline, ciprofloxacin, and amoxicillin residues [4,7,10], as well as gentamicin, ceftriaxone, and sulphadimidine residues [7,24] in milk by TLC, UHPLC, and MIT. However, data on the presence of various other residual antibiotics in dairy products are still limited. To address this gap, this study focuses on analyzing additional antibiotics, namely doxycycline, chlortetracycline, and enrofloxacin, along with tetracycline and oxytetracycline by reversed-phase high-performance liquid chromatography (RP-HPLC). As residual antibiotics are associated to potential health implications that often remain neglected, the current study aims to assess the risk using the hazard quotient in addition to the presence of residual antibiotics in milk samples. Furthermore, this study also focuses on establishing a correlation between these antibiotics, which have not been previously investigated in milk samples. The ultimate goal of the study is to raise awareness among relevant agencies, farmers, and policymakers regarding the proper utilization of antibacterial agents due to public health concerns.

2. Materials and methods

2.1. Study design

Milk samples were randomly collected from five different brands around Dhaka city between August and October 2022. This study included raw, soon-after-processed, marketed pasteurized, UHT, and flavored milk. Raw and soon-after-processed milk samples were collected from the milk processing plants located in Joydebpur, Mirpur, Narsingdi, and Narayanganj. On the other hand, various types

of marketed milk samples were randomly obtained from the retail stores in Dhaka city. Informed consent was obtained from all the milk processing plants before including them in the study and collecting their milk samples. The names of the brands were kept confidential, referring to A, B, C, D, and G. Additionally, two imported UHT milk samples were labeled E and F. The residual antibiotics in the milk samples were detected and analyzed using RP-HPLC, and the correlation between the occurrence of antibiotics was assessed.

2.2. Sample collection

A total of 27 samples, which included raw milk (n = 4), soon-after-processed (n = 4) milk, pasteurized milk (n = 5), UHT (ultra-high temperature) pasteurized milk (n = 5), and different flavored milk (mango, strawberry, chocolate, and kulfi) (n = 9) samples were collected for the study based on their availability and consumer preferences. Approximately 20 ml of milk samples were carefully transferred into sterile falcon tubes, ensuring proper labeling for each sample. The tubes were rapidly transferred into an ice box to the Food Analysis and Research Laboratory, Centre for Advanced Research in Sciences (CARS), University of Dhaka, and stored at −20 °C until analysis.

2.3. Chemicals and reagents

The chemicals and techniques utilized for the extraction and detection of residual antibiotic concentrations included Methanol (HPLC grade, Chromadolv® from Sigma-Aldrich), Acetonitrile (HPLC grade, Chromadolv® from Sigma-Aldrich), Deionized water, Oxalic acid (Loba Chemie Pvt Ltd, India), Trichloroacetic acid AR (Research-Lab Fine Chem Industries, Mumbai, India).

2.4. Equipment

The High-Performance Liquid Chromatography (HPLC) analysis was conducted using a SIL 20 series Prominence HPLC system (Shimadzu, Japan). The system consisted of an autosampler (Model SIL-20 AC), dual pumps (Model 20 AD), a column oven (Model CTO-20A), a vacuum degasser (Model DGU-20 A), and a UV–VIS detector (Model SPD-20 A). The LC solution software was employed as a component of the HPLC system for the analysis of residues. The particle separation was achieved by utilizing a Hypersil column with C_{18} selectivity (Supelco, USA). The column had dimensions of 250 * 4.6 mm and a particle size of 5 μm, with the column temperature being adjusted to 30 °C. Moreover, the vortex mixer (Digisystem VM-2000, Taiwan) and refrigerated centrifuge machine (Hettich Mikro 220R, Germany) were also used in this study.

2.5. Selection and preparation of standard antibiotics

Five commonly used veterinary antibiotics: Tetracycline (TC), Oxytetracycline (OTC), Doxycycline (DOX), Chlortetracycline (CTC), and Enrofloxacin (ENR) were considered. All the antibiotic standards were taken from Novartis (Bangladesh) Limited. These antibiotics, of high purity (at least 99 %), were used for measuring the residual concentration in milk samples. A stock solution was prepared by dissolving 10 mg of each reference antibiotic in 70 ml of a 5 % trichloroacetic acid (TCA) solution, 10 ml H_2O, and adding 20 ml of milk in a 100 ml volumetric flask, resulting in a concentration of 100 μg/ml. The solution was vortexed, centrifuged at 3000 rpm for 20 min, and then filtered. Subsequently, it was diluted with the same solvent to generate a standard working solution with varying concentrations (1.25–90 μg/ml), which were stored at 4 °C.

2.6. Blank sample preparation

The same procedure of standard preparation was also conducted with 20 ml of milk without antibiotics to obtain a blank result. Each sample underwent triple extraction, and each extract was injected three times into the HPLC.

2.7. Sample preparation

The milk sample extraction procedure followed the outlined protocols according to Ref. [25] with certain modifications. In this study, about 2 ml of milk samples were dissolved in 8 ml of 5 % TCA solution. The mixture was thoroughly shaken, followed by vortexing for 5 min and subsequent centrifugation at 3000 rpm for 20 min at room temperature. The supernatant was obtained and filtered by a 0.45 μm nylon syringe filter, which was then stored in an auto-sampler vial to facilitate subsequent chromatographic analysis. Lastly, about 20 μl of the sample eluate was injected into the HPLC system.

2.8. High-performance liquid chromatography (HPLC)

The chromatographic analysis was conducted, and conditions were followed as given by Ref. [26] with slight modifications. The chromatographic separation of residual antibiotics was achieved using the C_{18} Column, which was kept at 30 °C. The separation was conducted under isocratic conditions using a mobile phase consisting of an aqueous solution of oxalic acid (0.05 M) and acetonitrile in a ratio of 90:10 (v/v). The mobile phase was delivered through the system at a flow rate of 1.0 ml/min, and the entire duration of the run was maintained at 25 min. To achieve optimal sensitivity, quantitative measurements were conducted by choosing the proper

detection wavelength. Therefore, the absorption spectra were analyzed at a wavelength of 220 and 280 nm (UV). The detection wavelength of 280 nm was selected based on its higher peak intensity and maximum sensitivity.

2.9. Method validation

The HPLC method was standardized and validated using parameters including accuracy (% Recovery), precision (%RSD), linearity, the limit of quantification (LOQ), the limit of detection (LOD), tailing factor, and theoretical plates. Recovery analysis involved spiking 20 ml of blank milk samples with a suitable volume of standard antibiotics, followed by centrifugation and homogenization. Linearity was assessed by calculating six-point linear plots using three replicates and measuring the coefficient of determination (R^2). The matrix-matched calibrations were conducted within the concentration range of 0–15 μg/ml (0, 1.25, 2.50, 5.00, 10.00, and 15.00 μg/ml). Standard calibration curves were generated by plotting the analyte's peak area against its respective concentration in the unit of μg/ml. The LOD and LOQ were calculated from the calibration curve utilizing the equations given by the International Conference on Harmonization (ICH), which are: LOD = 3.3 Sa/b and LOQ = 10 Sa/b, here Sa = residual standard deviation, b = slope of the calibration curve.

2.10. Calculation of hazard quotient and risk assessment

The Hazard Quotient model was employed to analyze the risk associated with the consumption of milk containing residual substances. In this context, the hazard quotient is the ratio between the potential exposure to a substance and the concentration at which negative effects are not expected or predicted [23]. The hazard quotient is analyzed using the formula provided by Refs. [7,23]. Moreover, the Estimated Daily Intake (EDI) was computed using the equation outlined by Ref. [27]. After determining the residual antibiotics in milk samples, the risk estimation was conducted based on the mean concentrations of tetracyclines and enrofloxacin residues concerning the concentration recommended as an acceptable daily intake by JECFA [22]. EDI and ADI were computed to calculate HQ, which helped estimate the risk together. Moreover, the mean concentration and typical daily milk intake for adults and children with 60 kg and 10 kg body weights, respectively, were considered based on FAO/WHO guidelines [7,23]. Based on the report of the Department of Livestock Services, as of the 2022-23 fiscal year, Bangladesh has an average daily milk availability of 221.89 ml per capita. Furthermore, ADI represents an estimated amount of residue that can be safely consumed daily over a lifetime without discernible health risks, determined based on body weight. A hazard quotient equal to or less than one signifies minimal risk, whereas a value exceeding one signifies a significant potential risk to human health from exposure, considering it risky for the consumers, however, it does not represent the likelihood as per statistical chances [7].

2.11. Statistical analysis

IBM® SPSS® statistical package (version 26.0) and Microsoft Excel were used for the analysis. For each targeted antibiotic, descriptive statistics such as mean, standard deviation, %RSD, R^2, prevalence, graphs, and bivariate analysis (Chi-square test and Pearson's Correlation test) were computed.

3. Results

The method validation and standardization parameters, which yielded satisfactory results for accuracy, precision, and other performance parameters in analyzing TC, OTC, DOX, CTC, and ENR residues in milk samples, were conducted in accordance with the guidelines of the International Conference on Harmonization (ICH) and are summarized in Appendix A (**Table AI)** [28]. This analytical method also yielded symmetrical peak shapes with acceptable tailing factors[1] and an adequate number of theoretical plates.[2] Moreover, the calibration curves demonstrated strong linearity, confirming the reliability and suitability of the established methods for routine analysis, as illustrated in Appendix A (**Figure AI**).

The study findings revealed that all 27 milk samples tested positive for OTC residues, followed by CTC (29.63 %), ENR (22.22 %), and TC (18.52 %) residues. DOX residues were detected at the lowest level among the tested samples (Fig. 1).

Table 1 represents the concentrations of residual antibiotics in the milk samples. The mean concentration of TC residues was the highest among the positive samples, while the mean of OTC residues was the lowest compared to the other examined antibiotics. Moreover, the maximum concentration of both TC and OTC exhibited remarkably high levels compared to other antibiotics. In fact, the maximum concentration of all the tested antibiotics exceeded their MRLs.

Fig. 2 demonstrates the percentage of detected samples based on MRLs for antibiotic residues. A considerable proportion of the TC residues (80 %) exceeded the established MRLs, while half of the detected samples for DOX and CTC residues violated the limit. Although OTC residues were detected in all samples, only 22.22 % exceeded the limit.

Moreover, the screening outcomes for various milk categories are illustrated in Table 2. The presence of OTC residues was detected in both raw and soon-after-processed milk samples. Multiple antibiotic residues, including TC, DOX, CTC, ENR, and OTC, were

[1] For certain chemicals, the United States Pharmacopeia (USP) and Food and Drug Administration (FDA) mandates a tailing factor or peak symmetry factor of $\leq$2.0 [51,52].

[2] USP also specified theoretical plates $\geq$2000 as sufficient [53].

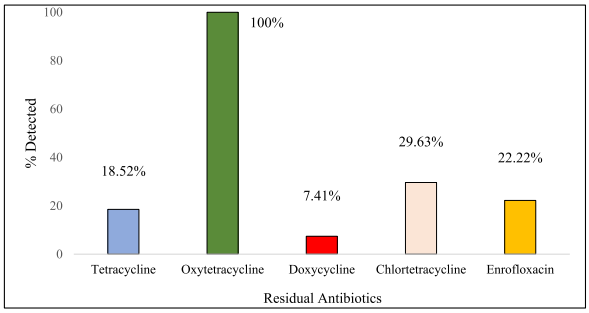

Fig. 1. Prevalence of residual antibiotics in milk samples.

Table 1
Concentration of residual antibiotics in detected milk samples.

Antibiotics	Detected samples n (%)	Maximum concentration (μg/l)	Minimum concentration (μg/l)	Mean (μg/l)	MRLs value (μg/l)
TC	5 (18.52)	357.84	65.10	196.40	100
OTC	27 (100)	315.10	20.03	87.66	100
DOX	2 (7.41)	102.88	83.56	93.22	100
CTC	8 (29.63)	188.91	51.57	116.46	100
ENR	6 (22.22)	116.38	64.83	94.49	100

Note. n = number of samples, and the values of MRLs (Maximum Residue Limit) were obtained from Refs. [29–31]. Tetracycline (TC), Oxytetracycline (OTC), Doxycycline (DOX), Chlortetracycline (CTC), and Enrofloxacin (ENR).

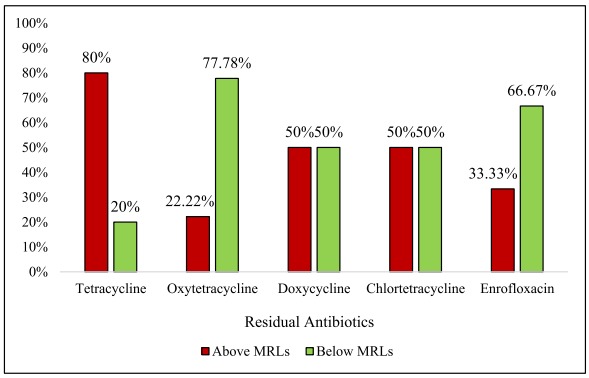

Fig. 2. Qualitative analysis of the detected samples concerning the MRLs.

detected in the majority of UHT and flavored milk samples, most of which exceeded the MRLs. Additionally, many pasteurized milk samples exhibited positive results for OTC residues. In the analyzed raw milk samples, no residues of OTC above the MRLs were detected. However, OTC residues were higher in the soon-after-processed milk samples. About 40.74 % (11 out of 27) of the milk samples contained residues of multiple antibiotics, and every examined milk sample exhibited the presence of OTC. The residual concentrations of the studied antibiotics in each milk sample are provided from Figure S1 to Figure S5 (**Supplementary data**). It was also determined that about 37.04 % of the positive milk samples (10 out of 27) exceeded the legal limit, while 44.44 % (12 out of 27) were very close to the upper limit of multiple antibiotics.

Fig. 3 (a), (b), (c)display the HPLC chromatograms of blank sample, tested standard antibiotics (OTC, TC, ENR, CTC, and DOX), and a positive milk sample indicating the presence of residual antibiotics, respectively. Evaluation of the blank sample in Fig. 3 (a) revealed the absence of any interference peaks originating from naturally occurring compounds in the milk matrix within the retention time. However, some interference peaks were seen before the retention time of the antibiotics. Moreover, the chromatograms of all the tested milk samples are provided in Fig. S6 (**Supplementary data**).

The correlation among the residual antibiotics is shown in Table 3. The study findings revealed a significant moderate positive linear correlation between TC and CTC, as well as a strong positive linear correlation between TC and ENR antibiotics. Furthermore, ENR antibiotics exhibit a significant positive linear correlation with TC, DOX, and CTC antibiotics. However, no correlation was observed between OTC and any of the other examined antibiotics.

Moreover, the Hazard Quotient (HQ) method was employed to evaluate and characterize the risk associated with dietary exposure across different age groups. Although the study results revealed lower HQ values for all antibiotics, the HQ value for TC was

Table 2
Overall prevalence of residual antibiotics in different milk samples.

Types of milk samples	TC n (%)	OTC n (%)	DOX n (%)	CTC n (%)	ENR n (%)	Overall positive	Samples above MRLs
Raw	0	4 (100 %)	0	0	0	4	0
Soon-after-processed	0	4 (100 %)	0	1 (25 %)	0	5	1
Pasteurized	1 (20 %)	5 (100 %)	0	1 (20 %)	1 (20 %)	8	2
UHT	2 (40 %)	5 (100 %)	2 (40 %)	1 (20 %)	1 (20 %)	11	2
Flavored	2 (22.22 %)	9 (100 %)	0	5 (55.56 %)	4 (44.44)	20	5

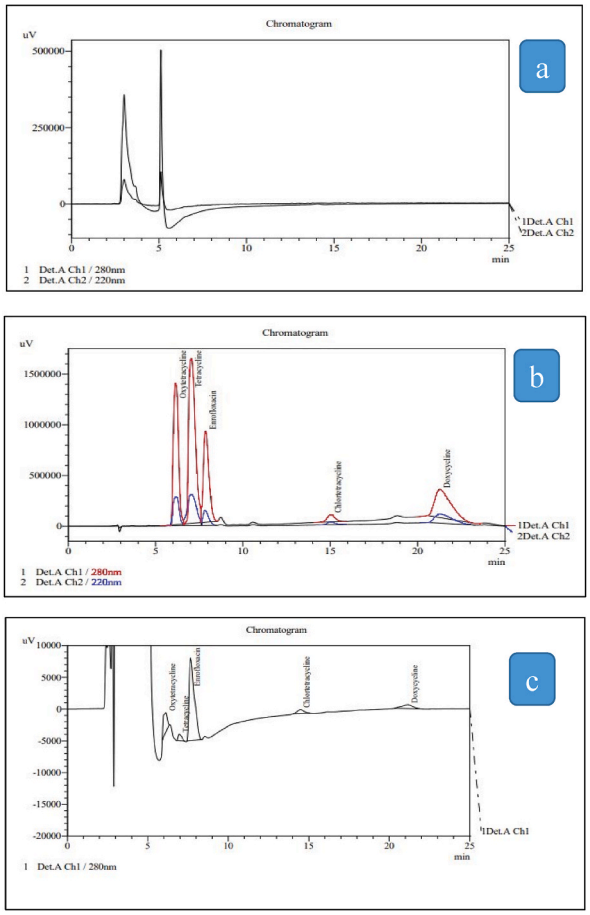

Fig. 3. HPLC Chromatograms for validation and standardization of OTC, TC, ENR, CTC, and DOX residues. **(a)** Chromatogram of the blank sample, **(b)** Chromatograms of all tested standard antibiotics (90 μg/ml) (highlighted 280 and 220 nm), and **(c)** Chromatograms of a positive sample demonstrating the occurrence of all antibiotic residues.

Table 3
Correlation of antibiotic residues among milk samples.

	TC	OTC	DOX	CTC	ENR
TC	1				
OTC	−0.015	1			
DOX	0.200	0.059	1		
CTC	0.487[b]	0.082	0.739[b]	1	
ENR	0.743[b]	0.028	0.406[a]	0.796[b]	1

[a] Correlation is significant at the 0.05 level (2-tailed).
[b] Correlation is significant at the 0.01 level (2-tailed).

comparatively higher than those of the other antibiotics. Additionally, children exhibited higher HQ levels for all detected residues compared to adults, indicating greater susceptibility in younger age groups (Table 4).

4. Discussion

In response to the rising concern surrounding antibiotic residues in milk and their associated health effects, few investigations have been conducted in Bangladesh in the last few years. Taking this into concern, the objective of the current study was to analyze residual antibiotics in various milk samples. The residual concentration levels reported in this study were higher than in previous investigations, which may be attributed to the arbitrary use of antibiotics and inadequate monitoring of particular antibiotic withdrawal periods. Our analysis detected traces of antibiotic residues in all of the analyzed milk samples (100 %). The occurrence of residual antibiotics in milk is influenced by the prevalence of diseases in dairy cows, treatment protocols, and different farming practices, which can vary across different study areas, ultimately contributing to the observed discrepancies in the results. Among the samples analyzed in our study, a high prevalence of OTC residues was observed, whereas DOX residues were detected only in two imported UHT samples. This is likely because DOX is prohibited for use in animals producing milk intended for human consumption [32]. However, evidence indicates that OTC antibiotics have been used for centuries, gaining high popularity, and previous studies revealed that 25–75 % of the applied (OTC) residues have the potential to remain in milk [33]. The presence of OTC residues in milk samples can be attributed to their random utilization in the treatment of cattle afflicted with clinical mastitis. As a result, this represents the most prevalent form of residual antibiotics observed in milk samples, which possess the capability to induce antibiotic resistance among pathogens [34].

Our study detected multiple antibiotics, which include OTC, TC, DOX, CTC, and ENR, in 40.74 % of the milk samples, with OTC residues detected in all samples. Raw and soon-after-processed milk samples contained only OTC residues, while marketed pasteurized, UHT, and flavored milk samples exhibited a higher prevalence of multiple residual antibiotics. The study also revealed that processed milk had higher OTC residue levels than raw milk samples. This could potentially be explained by a reduction in milk concentration resulting from the higher processing temperatures employed by dairy industries [35]. In addition to OTC, two commonly used medicines in the veterinary field are CTC and TC. These antibiotics have been observed to maintain their stability even at high and ultrahigh temperatures [36]. Similarly, ENR (fluoroquinolone) is employed as a therapeutic drug to treat diseases in cattle and has also shown a thermostable nature across various processing temperatures, attributed to its chemical structure [37]. Therefore, due to their thermal stability, residues of these antibiotics have been detected in heat-treated milk samples. Furthermore, the presence of multiple antibiotic residues in a single sample could be attributed to factors such as the administration or improper usage of medicated feed, concurrent parental or local administration of multiple drugs, inappropriate application, misuse of antibiotics, improper withdrawal periods, negligence of the farmers, veterinarians, and processors, and ineffective enforcement of laws [38]. Another important finding of the study was that the UHT milk from retail stores collected around Dhaka city possessed less amount of residues compared to the imported UHT samples.

Among the positive milk samples, the mean concentrations of TC, OTC, DOX, CTC, and ENR residues were 196.40, 87.66, 93.22, 116.46, and 94.49 μg/l respectively. These concentrations either exceeded the established MRLs of 100 μg/l [29–31] or were near the upper limit. The level of TC contamination reported in this study was higher than that found in another study conducted in Iran, which also quantified the mean residual concentration in different types of milk samples. The study found about 218.86, 6.63, 16.66, and 10.22 μg/kg of OTC, TC, CTC, and DOX residues respectively [15].

As our study did not encompass the entire milk supply chain, the higher prevalence of residual antibiotics in the pasteurized, UHT and flavored milk samples suggests a higher probability that the lactating cows providing the raw milk for these products had been subjected to improper antibiotics treatment or withdrawal periods before the collection period. Similar to the findings of this study, numerous previous studies detected residual antibiotics of the tetracycline group (TC, OTC, DOX, and CTC) in pasteurized milk and UHT milk samples. In a study conducted in Iran, about 19.78 % of the tetracycline residues were detected in pasteurized and sterilized milk samples [15]. Furthermore, a Brazilian study detected about 3 % residual antibiotics of the tetracyclines in commercial

Table 4
Characterizing the risk associated with dietary exposure to residual antibiotics through milk consumption across adults and children.

Antibiotics	Age Group	Body Weight (kg)	EDI[a] (μg/kg bw/day)	ADI[b] (μg/kg bw/day)	References	HQ[c]
TC	Adults	60	0.7263	30	[29]	0.02421
	Children	10	4.3579	30	[29]	0.14526
OTC	Adults	60	0.3242	30	[29]	0.01081
	Children	10	1.9451	30	[29]	0.06484
CTC	Adults	60	0.4307	30	[29]	0.01436
	Children	10	2.5841	30	[29]	0.08614
DOX	Adults	60	NA	NE	[31]	NA
	Children	10	NA	NE	[31]	NA
ENR	Adults	60	0.3494	372	[27]	0.00094
	Children	10	2.0966	372	[27]	0.00564

[a] Estimated Daily Intake (EDI) was calculated using the formula [(mean concentration of residue in milk x daily milk consumption per person)/ 1000]/ body weight.

[b] Acceptable Daily Intake (ADI) data are obtained from the Codex Alimentarius Commission and the European Agency for the evaluation of medicinal products, veterinary medicines, and inspections, NE= Not yet have been established.

[c] Hazard Quotient (HQ) was found using the formula EDI/ ADI, NA= Not available.

pasteurized milk samples [39]. A study in Poland also detected residues of tetracyclines (28.57 %) in fresh and UHT milk samples, with the level of residues being 17.73 % higher in fresh milk samples [40]. As our study detected high levels of residues in pasteurized milk samples, it can be claimed that the process of pasteurization could not disrupt the molecular orientation of antibiotics or their metabolites, indicating its existence in the finished products. This finding aligns with a study conducted in Brazil [41]. Moreover, improper application or inadequate duration of the UHT process, combined with the thermal stability of these compounds, may contribute to the high residue levels observed in the studied UHT milk samples [40]. In contrast to other studies conducted in Bangladesh, our research identified the presence of ENR residues (94.49 μg/l mean) in milk samples. While ENR residues have been commonly detected in previous studies conducted on chicken meat and poultry feed [42–45], their presence in milk had not been previously reported in Bangladesh. However, an investigation conducted in Egypt reported the presence of ENR residues in milk samples obtained from various sources, including markets, collection centers, and reservoirs, with mean concentrations of 2.94 μg/l [46].

About 37.04 % of the antibiotic-positive milk samples exceeded the established MRLs, where most of the positive samples were obtained from retail stores. Furthermore, most milk samples testing positive for TC residues violated the limit, while half of the positive samples with DOX and CTC residues exceeded the limit. Although the detected OTC residues in raw samples did not exceed the limit, their levels violated the limit in certain flavored milk samples or were very close to the upper limit (except Sample C) in processed samples. However, a study in Punjab, India, identified about 16 % of antibiotic residues, including enrofloxacin, oxytetracycline, tetracycline, and sulphamethoxazole, in raw milk samples, where only 4 % exceeded the limit. Similarly, another study of India also detected about 12.5 % of antibiotic residues in commercial milk ($n = 32$), with only 3.1 % violating the limit [47].

The Hazard Quotient (HQ), which evaluates health risks associated with the consumption of antibiotic residues in milk and the severity of their effects, was found to be less than one for the identified antibiotics in the milk samples. This indicates negligible adverse effects on consumer health from the examined milk samples. Findings revealed that the EDI for all detected antibiotics was quite below the ADI due to lower milk consumption per capita in Bangladesh, resulting in reduced exposure to milk residues. However, due to lower milk consumption, prolonged increased intake of milk and milk products could raise the risk of continuous residual antibiotic exposure. This persistence of residues, particularly in children whose primary nutrition source is milk, might lead to antibiotic resistance development in the future. Similar findings have been reported in studies [7,47] which concluded that there was no toxicological risk to consumers associated with the consumption of the tested milk samples containing the studied antibiotics. Nonetheless, since the ADI value for doxycycline was not determined for milk according to EMEA guidelines, its risk was not assessed [32].

Our study found a significant moderate to strong positive correlation between TC, CTC, DOX, and ENR antibiotics. Specifically, we observed that an increase in TC concentration was significantly correlated with an increase in CTC and ENR concentration, and an increase in DOX concentration was significantly correlated with an increase in CTC and ENR concentration. These findings suggest that the combined administration of these antibiotics in cattle may contribute to this association. For instance, CTC is often supplied in feed or water for preventative purposes, and TC is generally utilized for the short-term oral treatment of clinical illnesses [48]. Moreover, ENR is frequently used in treating mastitis, respiratory infections, and digestive system infections in cattle [49]. However, OTC did not demonstrate any significant correlation with the other antibiotics. This could be attributed to the ubiquitous presence of OTC in all of the analyzed samples, making it challenging to identify its potential correlation with other antibiotics.

The presence of residual antibiotics in milk can cause serious health hazards. Some precautionary approaches can be recommended to decrease their presence in milk, which include proper maintenance of the cattle rearing system, taking initiatives of the “One Health” approach, expansion of cattle farming extension programs among farmers, application of quality management protocols, administration of food safety laws and regulations by many relevant agencies, stringent prohibitions of random and misuse of antibiotics, the establishment of some guidelines regarding the withdrawal period, labeling of antibiotics, mode of administration, antibiotics marketing, maintenance of proper hygiene and sanitation, etc [50].

4.1. Strengths and limitations

The presence of antibiotic residues in milk poses a significant public health concern, influenced by numerous contributing factors. Detecting and quantifying these residues accurately and cost-effectively is essential. The current study utilizes HPLC-UV, a simple, reliable, economical, feasible, and widely proven method for detecting organic compounds in food samples. Additionally, the developed technique offers a practical solution that could serve as an effective method for the dairy industry to monitor these contaminants in the future. Furthermore, by including antibiotics such as doxycycline, chlortetracycline, and enrofloxacin, in addition to the commonly used tetracycline and oxytetracycline, this study addresses a gap in data regarding antibiotic residues in dairy products in Bangladesh. The study’s findings also provide valuable insights into the prevalence of these residues, assess the associated health risks, and emphasize the importance of proactive safety measures. However, resource constraints, including limited time, budget, and sample size, restricted the ability of the study to expand its scope across a broader geographical area and cover the entire dairy supply chain. It is important to acknowledge that the dairy industries in Bangladesh involves multiple processing facilities that collect raw milk from different districts and distribute pasteurized milk products nationwide, which requires substantial time and financial resources. The current study also did not explore the potential health impacts of antibiotic residues as it also needs a significant amount of time. Despite these limitations, the findings of the study cannot be ignored, as they emphasize the critical need for further research on antibiotic residues in milk to improve food safety and protect public health.

4.2. Future scope for research

For a comprehensive assessment of residual antibiotics in milk, future research should include a larger sample size that covers a wide range of farms, processing plants, retail outlets, and other establishments connected to the dairy industry. This would provide a more accurate understanding of the sources and farming practices contributing to high antibiotic residues in milk. Additionally, to evaluate the potential health impacts of these residues, such as the development of antimicrobial resistance or health risks linked to prolonged exposure, future studies could include in vivo testing. Such studies would explore the effects of various dosages and exposure durations of antibiotic residues, helping to determine their role in health issues like cancer and the emergence of antibiotic-resistant microbes.

5. Conclusion

The study findings revealed that oxytetracycline (OTC) was detected at the highest levels, followed by chlortetracycline (CTC), enrofloxacin (ENR), tetracycline (TC), and doxycycline (DOX) residues. The analysis also indicated that most marketed milk samples contained multiple residual antibiotics, most exceeding the maximum residue limits (MRLs). However, the hazard quotient for the identified antibiotics in milk samples was less than one, indicating negligible adverse effects on consumer health. Moreover, the estimated daily intake (EDI) of all detected antibiotics was well below the acceptable daily intake (ADI) due to low per capita milk consumption in Bangladesh, reducing exposure to milk residues. Nonetheless, prolonged increased intake of milk and milk products could raise the risk of continuous antibiotic exposure, potentially leading to adverse health effects. This study provides essential data for stakeholders to raise awareness about antibiotic residues in dairy products and suggests that expanding the research and creating a comprehensive milk supply chain database would enhance food safety and public health protection. The proposed validated and standardized analytical method can be a versatile tool for the food and drug industries to detect and identify multiple antibiotic residues in milk and other food samples.

CRediT authorship contribution statement

Farhana Rinky: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Asma Rahman:** Writing – review & editing, Formal analysis, Conceptualization. **Sompa Reza:** Writing – review & editing. **Abira Nowar:** Writing – review & editing. **Sharmin Rumi Alim:** Writing – review & editing, Conceptualization.

Ethics declarations

Review and/or approval by an ethics committee was not needed for this study, as it did not involve any human subjects or live animals. All milk samples were collected from collection centers, processing plants, and retail shops, which excluded us from handling animals.

Consent for publication

All authors have approved the publication of this manuscript.

Data availability statement

Data will be made available upon reasonable request to the corresponding author.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e41422.

Appendix A. Method validation parameters

Table A1
Method validation parameters for the determination of TC, OTC, DOX, CTC, and ENR residues in milk by RP-HPLC

Parameters	TC	OTC	DOX	CTC	ENR
Linear equation	y = 69224x + 415.83	y = 59663x + 841.06	y = 52174x + 1744.4	y = 32511x + 3337.3	y = 71485x + 6615.7
R^2	0.9999	0.9999	0.9999	0.9997	0.9990
Linearity range	0–15 μg/ml				
Theoretical plates (n = 5)	6615.961	5314.452	5918.732	6945.167	6561.888
Tailing factor (n = 5)	1.669	1.573	1.567	1.769	1.367
Resolution (n = 5)	2.955	0.000	7.583	12.751	2.058
Precision (intra-day, n = 6) (% RSD)	0.33	0.33	0.33	0.65	0.38
Precision (inter-day, n = 6) (% RSD)	0.49	1.33	0.60	0.38	0.28
% Recovery (mean ± SD)	99.25 % ± 0.26	100.39 % ± 0.41	101.21 % ± 0.52	99.75 % ± 1.00	100.75 % ± 0.32
LOD (μg/ml)	0.034	0.034	0.033	0.060	0.027
LOQ (μg/ml)	0.110	0.112	0.109	0.198	0.089

Note. Equation of calibration curve, y = mx + c, n = number of determinations, R^2= Coefficient of Determination ($0.9995<R^2<1$), LOD = Limits of detection, LOQ = Limits of quantification, % RSD = Percent Relative Standard Deviation (≤2), and % Recovery (with 95–105 %) [28].

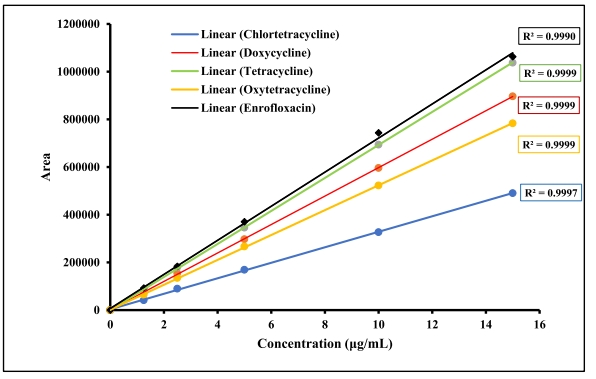

Fig. A1. Linear curve for the standard antibiotics

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