Heliyon 7 (2021) e07739

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Determination of antibiotic residues in milk and assessment of human health risk in Bangladesh

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ARTICLE INFO

Keywords:
Antibiotic residue
Human health
Milk
Risk assessment
TLC
UHPLC

ABSTRACT

Consumption of milk contaminated with antibiotic residues above the maximum residue limit (MRL) causes toxicity to humans and the development of superbugs that leads to the failure of antibiotic therapy and threatens human life. Moreover, long-duration exposure might alter the nature of gut microflora, resulting in the enhancement of many diseases. Therefore, our study aims to find out the residues level of selected antibiotics in milk and assessments of humans health risks. We examined 300 raw and processed milk samples using thin-layer chromatography (TLC) and ultra-high-performance liquid chromatography (UHPLC) methods against five veterinary antibiotics and assessed the health risk for consumers in Chattogram, Bangladesh. Risk analysis was done by using a hazard quotient based on 165 ml per capita milk consumption. We found a total of 7 % prevalence of antibiotic residues in raw milk, which were higher (8 %) in individual milk samples than the pooled samples (4 %). However, we did not find any antibiotic residues in processed milk. The mean concentration of oxytetracycline residue was detected at 61.29 μg/l, and amoxicillin was 124 μg/l in individual milk samples. Risk analysis showed that, the hazard quotient values are 0.0056 for oxytetracycline and 0.0017 for amoxicillin residues. This result implied no significant health risks associated with the consumption of milk produced and marketed in the study area. Our study might fill up the gaps of knowledge in measuring the safety status of milk regarding public health issues.

1. Introduction

Antibiotic resistance has gained a global health concern as it is attributed to the death of about 0.7 million people every year, which is expected to rise to 10 million per year in 2050 [1,2]. The use of antibiotics in food-producing animals causes the subsequent deposition of these drug residues in milk, meat, and eggs [3, 4, 5, 6]. The residues then lead to developing antimicrobial-resistant bacteria in animals and releasing them into the surrounding environment and on different animal-originated food items [7]. Thus, antibiotic uses in food animals facilitate AMR infection in humans [8]. Furthermore, antibiotic residues contaminated the environment to pose an ecological risk for resistant bacteria that might threaten public health [9]. Food of animal origin was responsible for the resistance of different bacteria in humans, especially *Staphylococcus* spp., *Salmonella* spp., and *Campylobacter* spp [7]. However, there is still a lack of data and literature on the use of antimicrobials in the food animal production system and the development and spread of resistance to humans [10]. Antibiotics are used in animals to treat clinical diseases, as prophylaxis to prevent diseases, and as non-therapeutic to enhance animal growth [11]. Food and Drug Administration (FDA) reported that around 80 % of all antimicrobials in the agricultural sector are destined for food-producing animals [12]. After administered, a proportion of antibiotics or their metabolite accumulated and deposited within various cells, tissues, and organs of the body that remain pharmaceutically active is called antibiotic residues [13]. Poor sanitation, hygiene, mismanagement of antibiotics in the farm, and irrational use lead to higher residues. These residues might be present in different consumable food products of animal origin like milk, meat, egg and skin during the withdrawal period which is specific for different groups of antimicrobials [14]. Around 40–90 % of administered antibiotics is excreted through urine and feces as active form, leads to environmental contamination [15]. Some antimicrobials such as erythromycin, sulfamethoxazole, and tetracyclines can persist in soil and water for

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https://doi.org/10.1016/j.heliyon.2021.e07739
Received 3 November 2020; Received in revised form 18 April 2021; Accepted 5 August 2021

a period of more than a year [16]. In environment, the antibiotic residues promote the development of resistant bacteria through selective pressure [17]. Subsequently humans usually are infected through antibiotic resistant bacteria due to poor sanitation and hygiene. It is also evidenced that veterinary antibiotic residues stored in various plant parts including leaves stem, and roots [18]. In dairy cows, a wide range of antibiotics is used to treat and prevent mastitis (udder infection), which is more prevalent. Many of these residues are not neutralized by the conventional heat treatment used for manufacturing pasteurized milk (72 °C) in industries [19]. It creates a problem for the preparation of fermented dairy products like cheese, dahi, and yogurt by partially or fully inhibiting the growth of lactic acid-producing bacteria in starter cultures [20]. Globally, milk and milk products with antibiotic residues above the maximum residue limit (MRL) and resistant bacteria are recognized as a threat to public health [8]. MRL value was established by the different regulatory bodies and defined as the maximum concentration of a residue that is legally permitted and recognized as acceptable in food [21]. Exceeding the MRL might favor the growth of resistant pathogenic bacteria harmful to animals, humans, and the environment. Antibiotics residues are extracted out through urine after dietary consumption of animal-originated foods by humans and contaminated the environment and water sources [22, 23, 24]. Moreover, studies in animal models proved that sub-therapeutic antibiotics or residues altered consumers' microbiota composition and metabolic phenotypes. More importantly, antimicrobial residues affect the ileal expression of genes involved in immunity and the body mass of the host [25, 26]. Residues consumed through milk may cause different harmful reactions to human health like carcinogenic, mutagenicity, teratogenic, nephropathy, reproductive disorders, hepatotoxicity, and allergic reactions [27]. In addition, penicillin residue is a potential cause of urticaria even in a meager amount [28]. Moreover, severe illness related to skin irritations due to antibiotic-contaminated milk was reported, but the anaphylactic shock is not well documented [29].

Different analytical methods have been developed to examine the drug residues in milk, divided into screening tests and confirmatory tests. Screening methods are qualitative-based methods like thin layer chromatography and microbial inhibition test usually used to detect residues [30]. In contrast, confirmatory methods are costly and require more time and trained personnel. Methods like Liquid Chromatography (LC) coupled with different detection modes like mass spectrometry (MS) and UV [31], High-Performance Liquid Chromatography (HPLC), and Capillary electrophoresis (CE) are commonly used as confirmatory methods in quantitative research [32]. HPLC contains various mobile phases, an extensive library of column packings, and variations in modes of operations [33].

The probability of potential adverse health effects caused by antimicrobial residue can be measured by calculating the risk assessment. Generally, chemical risk assessment consists of four well-defined steps-hazard identification, hazard characterization/dose-response assessment, exposure assessment, and risk characterization [34]. Hazard quotient (HQ) and risk quotient (RQ) are two widely used concepts of chemical risk assessment. The hazard quotient is used for health risk assessment, while the risk quotient is applied in ecological risk assessment. Risk assessment is highly preferred for the maintenance of food safety to ensure public health.

Due to the growing concern over antimicrobial resistance and food safety issues in Bangladesh, few unstructured studies were done focused on the detection of antibiotic residues in milk, meat, and eggs. However, the chance of public health risk for dietary exposure to antimicrobial residues remains undetected. Therefore, we evaluated the prevalence of antibiotic residues in milk and assessed associated human health risks in Bangladesh.

2. Methodology

2.1. Ethical approval

The research was conducted by following the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Chattogram Veterinary and Animal Sciences University (CVASU-EC), Bangladesh [permit number: CVASU/Dir (R and E) EC/2019/126 (02), Date: 29/12/2019]. However, the consent from all the subjects have been taken appropriately before inclusion of them in the study.

2.2. Study design

We conducted a cross-sectional study in Chattogram city and nearby Patiya Upazila (sub-district) under the Chattogram district, Bangladesh, from February to August 2019. Chattogram is the second-largest city in Bangladesh, and the Patiya Upazila is considered the milk production pocket where dairy farming is entirely developed to fulfill the city's demand. We have included both raw and processed packet milk in samples in this study. Raw milk samples were constituted of individual and pooled samples managed from different dairy farms under the study area. TLC test was applied for screening of all samples against common antibiotics. Then, based on our resources, only positive samples containing amoxicillin and oxytetracycline residue were analyzed by UHPLC to measure the antibiotics residue concentration. We also performed a risk assessment for the detected concentration by hazard quotient formula.

2.3. Sample collection

Individual samples were collected from a few purposively selected milking cows depending on the herd size of each farm. Pooled samples were taken from the milk storage tank after milking the herd. On the other hand, different varieties of processed packet milk like pasteurized, UHT (pasteurized with ultra-high temperature), mango, chocolate, and strawberry milk were purchased from different markets within Chattogram city (See Table 1). About 20 ml of milk was filled in a falcon tube with proper labeling for each sample and immediately carried to the clinical pathology laboratory of Chattogram Veterinary and Animal Sciences University (CVASU) inside a cool box. Finally, all samples were stored in a deep freezer at -20 °C and analyzed within 48 h. Time between sample collection and placed in a -20 °C ranged within 30–45 min.

2.4. Selection and preparation of antibiotics

We considered five commonly used veterinary antimicrobials: Amoxicillin, Gentamicin, Ceftriaxone, Oxytetracycline, and Streptomycin to screen milk samples by the TLC. All these antimicrobial standards were purchased from Sigma Aldrich (Fluka and Vetranal), Company, USA, and prepared for comparison with the extracted samples. We followed the standard operating procedures for storing and handling standards. Stock solutions were prepared by dissolving 0.1 gm of standard in 2 ml of methanol. This solution was further diluted using the same solvent to make a standard working solution of different concentrations and stored in a deep freezer at -20 °C.

2.5. Thin layer chromatography (TLC)

About 1 ml of milk was added with 1 ml of acetonitrile-methanol-deionized water at a ratio of 40:20:20 in a centrifuge tube. After mixing correctly, the mixture was centrifuged at 3000 rpm for about 10 min. Then, the supernatant was used in the TLC method described by [35]. Positive samples were stored at −20 °C for UHPLC analysis.

2.6. Ultra high-performance liquid chromatography (UHPLC)

We optimized a UHPLC-DAD (High-Performance Liquid Chromatography-Diode Array Detector) technique for detecting two antimicrobials residue-oxytetracycline and amoxicillin at sub MRL levels. The method was validated for specificity, precision, recovery, and linearity. The extracted samples were centrifuged for 15 min at

Table 1. List of processed milk samples of different brands tested in the study.

Sample categories	Different brands available in market								
	Pran	Arong	Milkvita	Farm & fresh	Nahar	Mugal	Starship	Milk man	Total samples
Pasteurized	7	7	7	7	7				35
UHT	7	7	7	7		7			35
Mango milk		5						5	10
Chocolate milk		5					5		10
Strawbery milk		5					5		10
									100

3000rpm in the Eppendorf tube, followed by filtration using 0.2nm MFS filters. The final extraction of samples was set to run in the UHPLC by the procedure described for amoxicillin [36] and oxytetracycline [37]. A stainless column C 18 (P/N 891 - 5002, 2 mm ID×10 0 mm, L No. 22G2C - 001) was used for chromatography in all cases. The mobile phase was pumped at a flow rate of 0.2 ml/min for amoxicillin and 1.5 ml/min for oxytetracycline. In the case of amoxicillin, the analytes were detected at 254 nm wavelength, whereas it was 360 nm for oxytetracycline. Injection volume for both amoxicillin and oxytetracycline was 20 µl in the UHPLC system.

2.7. Method validation

Recovery, precision, the limit of quantification (LOQ), and the limit of detection (LOD) were used to standardize and validate the UHPLC system [38]. For the recovery analysis, 5ml of blank milk samples were spiked with the centrifuged antibiotic standards to obtain various concentrations: 50, 100, and 150 ppb. Then the samples were going through the clean-up procedure. To estimate the Limit of Quantification, samples were spiked with 0.01 µg/ml. The limit of quantification (LOQ) was calculated at a signal-to-noise ratio of 10. The limit of detection (LOD) value was calculated at a signal-to-noise ratio of 3. Blank determinations were also analyzed to calculate the LOD and LOQ values.

2.8. Estimation of hazard quotient and risk assessment

We used the Hazard Quotient model to assess the risk of consuming residues with milk. The hazard quotient (Eq. (1)) is the ratio of the potential exposure to a substance and the level at which no adverse effects are expected.

$$\text{Hazard Quotient} = \text{Estimated daily intake / accepted daily intake} \quad (1)$$

The estimated daily intake (EDI) was calculated (Eq. (2)) by the following given equation [39].

$$\text{EDI} = (\text{concentration of residue as µg/kg}) \text{ x } (\text{daily intake of food in kg/person}) / \text{Adult body weight (60 kg)} \quad (2)$$

The mean level of antibiotic concentrations in raw milk was calculated. Then the value of the mean concentration and average daily milk consumption based on 60 kg body weight were taken into consideration. According to the data provided by the Directorate of Livestock Services (DLS), the per capita availability of milk in Bangladesh was 165.07 ml/day [40].

Acceptable Daily Intake (ADI) is an estimated amount of residue allowed for ingested daily over a lifetime without any appreciable health risk expressed on a bodyweight basis. ADI of amoxicillin and oxytetracycline is 0.2 and 0.03 mg/kg body weight/day, respectively [41].

Table 2. Comparative prevalence of antimicrobial residues in two study areas.

Location	sample	Total sample	positive	% of positive (95 % CI)
Chattogram city	Individual	97	10	10.30 (3–14.3)
	Pooled	40	2	5 (0.6–16.9)
Patiya Upazila	Individual	53	2	3.77 (0.5–13)
	Pooled	10	0	0 (0.0–30.8)

Table 3. Overall prevalence of different antibiotics residue in milk.

Categories of milk samples (N)	Antibiotic tested					Overall positive	Overall percentage (95 % CI)
	Amoxicillin (n, %)	Oxytetrcycline (n, %)	Streptomycin (n, %)	Gentamicin (n, %)	Ceftriaxon (n, %)		
Pooled samples (50)	0	0	0	2 (4 %)	0	2	4 (0.5–13.7)
Individual samples (150)	3 (2 %)	5 (3.33 %)	2 (1.33 %)	1 (0.6 %)	1 (0.6 %)	12	8 (4–13)
Processed samples (100)	0	0	0	0	0	0	0 (0.0–3.6)
Total (300)						**14**	**4.6 (2.5–7.7)**

Table 4. Concentration of oxytetracycline and amoxicillin residues in individual milk samples.

Antibiotics	No of positive samples	Maximum concentration (µg/l)	Minimum concentration (µg/l)	Mean ± SD (µg/l)	MRL value[a] (µg/l)
Oxytetracycline	5	116	6.57	61.29	100
Amoxicillin	3	345	5.85	124	4

[a] The value of the MRL (maximum residue limit) was collected from the Codex report [21].

A hazard quotient less than or equal to one indicates negligible hazard, while greater than one states the likelihood of harm. However, it does not indicate the statistical probabilities of occurrence.

3. Results

TLC results represented a total prevalence of 7 % (14 out of 200) in raw milk samples and 0 % in processed packet milk samples. Screening results for different categories of samples are shown in Table 2 and Table 3. In all positive pooled samples, only the gentamicin residue (4 %) was found. Individual milk samples were recognized as positive against all five tested antibiotics in the following percentages- Amoxicillin (2 %), oxytetracycline (3.3 %), streptomycin (1.3 %), gentamicin (0.6 %), and ceftriaxone (0.6 %)

The results of the UHPLC analysis are presented in Table 4. The oxytetracycline standard concentration was 520 μg/l, with a recovery time of 6.087 min and a peak area of 38953708. The concentrations in five positive samples were found to be 26.15 μg/l, 6.57 μg/l, 49.73 μg/l, 116 μg/l, and 108 μg/l. The UHPLC Chromatogram for validation and standardization of oxytetracycline residues, the standard, blank milk sample, and positive sample with standard were presented in Figure 1 (a), (b) and (c).

In the case of amoxicillin, the value of standard was 200 μg/l and the recovery time was 3.7 min with a peak area of 2143200. The three positive samples contained residues as 345 μg/l, 21.5 μg/l, and 5.85 μg/l. The UHPLC Chromatogram for validation and standardization of amoxicillin residues, the standard, blank milk sample, and positive sample with the standard was presented in Figure 2 (a), (b), and (c).

Based on the mean value of residues, the Hazard Quotient was calculated to characterize the risk of dietary exposure to oxytetracycline and amoxicillin through the milk. The results are shown in Table 5.

4. Discussion

We determined the prevalence of antibiotics residue in milk. Our results in raw milk samples were lower when compared to the total prevalence found in two previous studies conducted in Chattogram who found 18 % and 18.6 %, respectively [3, 42]. Variation may occur due to the efficiency of the tests used for the screening and study time. We used the TLC method, whereas other studies used microbial inhibition tests and commercial kits. Different factors like sample size, location, and duration also lead to the fluctuation in results. Moreover, the use of antimicrobials in dairy cows and found residues in milk dramatically depends on the disease burden of the study area, which may have contributed to the different results.

We found a relatively higher prevalence of antibiotic residues in the individual milk samples than pooled samples. It may be due to the collection of pooled samples from milk storage tanks where contaminated milk became mixed with a considerable volume of pure milk coming from healthy cows of the farm. So, the concentrations of the residues were diluted at an undetectable level. However, as the individual samples were taken separately from each cow, it possessed a high level of residue if the cows were treated with antibiotics before the sample collection period. We detected all of the tested antibiotics-oxytetracycline, amoxicillin, streptomycin, ceftriaxone, and gentamicin in individual milk samples. A study in Iran found that 19.78 % of the milk samples were contaminated with tetracycline residues [43], while another study in Pakistan showed 36.5 % positive for Beta-lactam antibiotic residues in the unprocessed market milk [44]. Our results of amoxicillin and oxytetracycline residues were lower than the previous finding in the area [42], and this was might be due to the difference in the time duration of the study. In recent years farmers are more educated and aware of antimicrobial uses in their farms.

On the other hand, farm management and biosecurity are more improved in the country now than earlier. In pooled samples, only gentamicin residues were detected. It might be due to using a high dose of gentamicin for treating mastitis in dairy cows in the study areas.

None of the processed milk samples were recognized as positive for selected antibiotics in our study because market milk samples were pasteurized (heat-treated) with continuous sterilization and aseptic filling. A few were prepared by ultra-high temperature (UHT) technology, which requires temperatures of at least 135 °C for a period of up at least one second (usually 3–5 s). Studies found that pasteurization practices lead to the reduction of antimicrobial residues in milk [45]. The processed milk was marketed by the companies that collected raw milk from different dairy farms and monitored appropriate standard and stewardship to maintain the quality of their brands. Another critical factor was the variation in our packet milk samples, including mango milk, chocolate milk, and strawberry milk containing minimal milk. Our result was concordant with a previous investigation which tested 94 UHT milk and found no samples containing detectable levels of antibiotics, namely tetracyclines [46]. One study in Iran recognized that 7.8 % of samples were contaminated with oxytetracycline and tetracycline residues in pasteurized milk samples, but all concentrations were below the MRL value [47]. Previous literature also stated that processed milk had a lower percentage of antimicrobial residues than raw milk [20, 48].

In our study, the average concentration of amoxicillin residue in individual raw milk samples was detected several times higher than the MRL (4 μg/l) set by Codex Alimentarius Commission (CAC), and this finding was supported by a previous researcher [49] who also observed amoxicillin residues in raw milk above the MRL. However, their concentrations had been up to 53.7 μg/l, which was lower than our result. We also measured the mean value of oxytetracycline residue in milk lower than the MRL (l00 μg/1) even though two of the five positive samples crossed the MRL value. Our mean value was lower than the previous results [50, 51], which stated 149.4 μg/l and 150 μg/l respectively. Another study carried out in Iran also recognized 945.90 μg/l of oxytetracycline residue in milk [43]. Detection of lower concentration may be caused by limited use of oxytetracycline in dairy farms due to the availability of new generation antimicrobials.

Cattle rearing system and waste management are critical for developing and transmitting antibiotic-resistant pathogens from animals to the environment and humans [52]. Implementation of strict biosecurity practices on dairy farms, improved hygiene and welfare, and following proper antibiotic stewardship could control antibiotic residues in the products and reduce humans health hazards [53].

The Hazard Quotient expresses the risk posed to human health by consuming milk having residues and presents the intensity of the hazardous effect. Results revealed that the estimated daily intakes (EDI) were much lower than the acceptable daily intakes (ADI) for amoxicillin and oxytetracycline. On the other hand, the less per capita milk consumption of Bangladeshi people contributes to lower exposure to residues found in milk. The hazard quotient values below one proved that detected levels of residues in milk had no significant toxicological effects on the health of consumers in the study area. Similarly, the calculated EDI based on the 200 ml average daily milk consumption in Macedonia was found 2 to 100 times lower than the values of the acceptable daily intakes stated by the World Health Organization [50]. Another recent study on milk in Croatia reported that the estimated dietary exposure against the detected concentration of amoxicillin, ampicillin, benzylpenicillin, cloxacillin, cephapirin, cefazolin, cefoperazone, and ceftiofur was not exceeding the acceptable daily intake [54]. Likewise, daily intake of residues (EDIs) for the average daily milk consumption of 300 ml was 20–1640 times lower than the values of acceptable daily intakes (ADIs) based on the European Medicines Agency and World Health Organization [55]. A study in India also presented the hazard quotients for oxytetracycline as 0.009, indicating negligible public health risk [56].

The limitations of our study were a short duration of time and a low resource laboratory setting. It will require a couple of years to reveal the comprehensive status of different antibiotic residues in various milk samples throughout the year and measure associated risks for consumers health. We have established UHPLC settings for only two veterinary antibiotics-amoxicillin and oxytetracycline residue quantification. To the

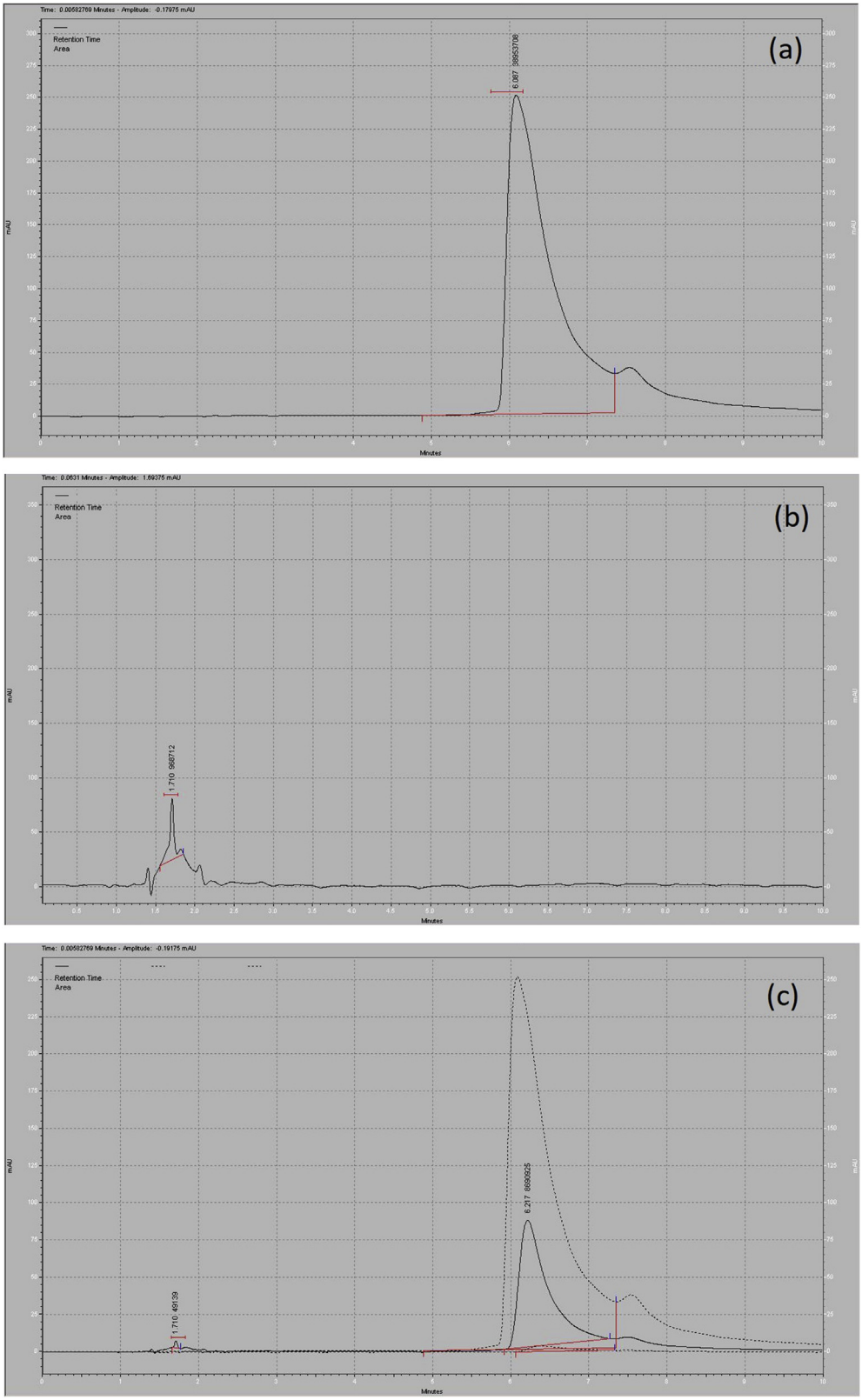

Figure 1. UHPLC chromatograms for validation and standardization of oxytetracycline residue. (a) Chromatogram of oxytetracycline standard (520 μg/l), (b) Chromatogram of blank milk sample, and (c) Chromatogram of oxytetracycline positive sample (concentration: 116 μg/l) with standard.

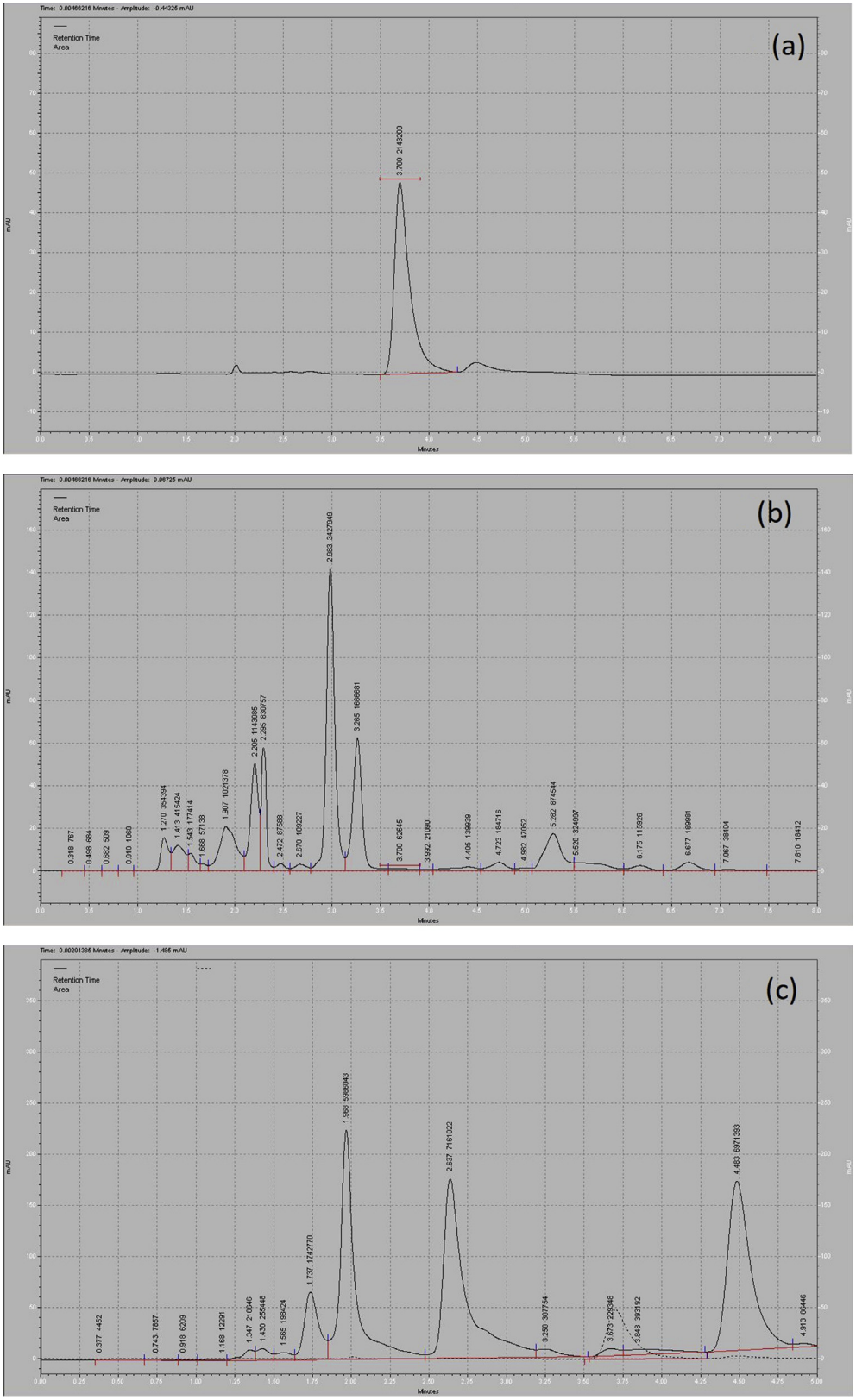

Figure 2. UHPLC chromatograms for validation and standardization of amoxicillin residue. (a) Chromatogram of amoxicillin standard (concentration 200 μg/l), (b) Chromatogram of amoxicillin negative sample, and (c) Chromatogram of amoxicillin positive sample (concentration 21.4 μg/l) with standard.

Table 5. Estimation of risk assessment by Hazard Quotient for mean concentration[a] of residues in raw milk.

Antibiotic	EDI[b] (μg/kg/day)	ADI[c] (μg/kg/day)	Hazard Quotient
Oxytetracycline	0.168	30	0.0056
Amoxicillin	0.341	200	0.0017

[a] The mean concentration of oxytetracycline and amoxicillin residue was 61.29 μg/l and 124 μg/l, respectively.
[b] Estimated daily intake (EDI) was calculated by the following formula (milk consumption * mean concentration of residue in milk)/body weight (60kg).
[c] Acceptable daily intake data derived from the Australian Pesticides and veterinary medicines authority [41].

best of our knowledge, in Bangladesh, this is the first study that introduced the risk assessment approach for drug residues present in milk.

5. Conclusions

Although most of the milk samples of the study area possess veterinary antibiotics residue above the MRL value, it would not be detrimental to humans health following consumption. This study provides the baseline information for policymaking and extended investigator, especially for risk analysis to protect public health threats. Extending this study and developing a database for the concentrations of different antibiotic residues and associated risk levels might facilitate the effort to ensure food safety and public health.

Declarations

Author contribution statement

Md. Sahidur Rahman: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammad Mahmudul Hassan: Performed the experiments; Wrote the paper.

Sharmin Chowdhury: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This work was supported by Bangladesh Agricultural Research Council (BARC) (PIUBARC, NATP-2 subproject ID 138).

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors express thanks to Yasir Hasib, Jahan Ara, and Md. Sirazul Islam (post-graduate students of CVASU) for their assistance in sample collection.

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