Determination of Antibiotic Residue in Milk and Assessment of Human Health Risk in Bangladesh

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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. Besides, long-duration exposure might alter the nature of gut microflora results in the enhancement of many diseases. Therefore, we examined 300 raw and processed milk samples using thin layer chromatography (TLC) and ultra high performance liquid chromatography (UHPLC) method against five veterinary antibiotics and assessed the health risk for consumers in Chattogram, Bangladesh. Risk analysis was calculated by using hazard quotient on the basis of 165 ml per capita milk consumption. We found a total of 7% prevalence of antibiotic residues in raw milk samples which were higher (8%) in individual samples than the pooled samples (4%). However, we did not find any processed milk samples as positive. The mean concentration of oxytetracycline residue was detected 61.29 µg/l and amoxicillin was 124 µg/l in individual milk samples. Risk analysis showed the hazard quotient values of 0.0056 for oxytetracycline and 0.0017 for amoxicillin residue which was confirmed that, no significant health risk associated with the consumption of milk produced and marketed in the study area. Our study might fill the gap of knowledge to measure the safety status of milk regarding public health issues.

Keywords: antibiotic residue, human health, milk, risk assessment, TLC, UHPLC

Introduction

Antibiotic resistance has gained a global health concern through the record of about 0.7 million people killed every year around the globe and this number could rise to 10 million per year in 2050 [1]. Irrespective use of antibiotics in food animals and subsequent release of these drug residues through milk meat and egg is a leading cause development of antibiotic resistance in the human body. Antibiotics are used in food animals to treat clinical disease, to prevent diseases as well as enhance animal growth. Food and Drug Administration reported that around 80% of all antimicrobials of agricultural sector are destined for food-producing animals [2]. After administered a proportion of these drugs or their metabolite accumulated, deposit or store within various cells, tissues and organs of the body which remain pharmaceutically active is called the residue of this drug [3]. These residues might present different consumable food products of animal origin like milk, meat, egg and skin [4] during the withdrawal period which is specific for different groups of antimicrobials. Besides, these residues excreting through urine and feces lead the environmental contamination. In dairy cows' wide range of antibiotics used specially to treat and prevent mastitis (udder infection). Many of these residues are not neutralized by the conventional heat treatment used for manufacturing pasteurized milk (72^0C) in industries [5]. It also creates a problem for the preparation of fermented dairy products like cheese, dahi and yogurt by partially or fully inhibits the growth of lactic acid-producing bacteria in starter cultures [6]. Milk having antibiotic residues above the maximum residue limit (MRL) is recognized as harmful for human health worldwide [7]. 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 a food [8].

Different analytical methods have been developed to examine the drug residues in milk which can be divided as a screening tests a and a confirmatory test. Screening methods are qualitative based, usually used to detect the presence of residues like thin layer chromatography and microbial inhibition test [9]. Confirmatory methods are highly expensive and require more time and trained personnel. Liquid Chromatography (LC) coupled with different detection modes like mass spectrometry (MS) and UV [10], High Performance Liquid Chromatography (HPLC) and Capillary electrophoresis (CE) are commonly used in quantitative research [11]. HPLC contains characteristics like a variety of mobile phases, extensive library of column packings and variation in modes of operations. [12]

The probability of potential adverse health effects caused by antimicrobial residue can be measure by calculating the risk assessment. Generally, chemical risk assessment consist of four well-defined steps-hazard identification, hazard characterization/dose-response assessment, exposure assessment and risk characterization [13]. Hazard quotient (HQ) and risk quotient (RQ) are two widely used concepts of chemical risk assessment. Hazard quotient used for health risk assessment while the risk quotient applied in ecological risk assessment. This approach is highly preferred for the maintenance of food safety to ensure public health.

Due to growing concern over antimicrobial resistance and food safety issues in Bangladesh, recent studies focused on the detection of antibiotic residue in milk, meat and egg. However, the importance of risk assessment for dietary exposure of antimicrobial residues remained neglected. In this context, we evaluated the risk assessment along with the prevalence of residue in Chattogram city and Patiya Upazila of Bangladesh. This study provides the baseline information for policymaking and extended investigation especially for risk analysis to protect public health.

Methodology

Study design:

We conducted this cross-sectional study in Chattogram city and nearby Patiya Upazila (sub-district) under Chattogram district, Bangladesh within the period of February to August, 2019. Chattogram is the second largest city of Bangladesh and the Patiya Upazila is considered as a milk production pocket where dairy farming is quite developed to fulfill the demand of the city. 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 only positive samples containing amoxicillin and oxytetracycline residue were analyzed by UHPLC to measure the concentration of residue. We also performed a risk assessment for the detected concentration by hazard quotient formula.

Sample collection

Individual samples were collected from a few purposively selected milking cows depends on the herd size of each farm. Whereas, 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. About 20 ml of milk was filled in a falcon tube with proper labeling for each sample and immediately carried to clinical pathology laboratory of Chattogram Veterinary and Animal Sciences University (CVASU) through a cool box. Finally, all samples were stored in a deep freezer at -20^0 C and analyzed within 48 hours.

Selection and preparation of antibiotics

We considered five commonly used veterinary antimicrobials-Amoxicillin, Gentamicin, Ceftriaxone, Oxytetracycline (OTC) and Streptomycin for the screening of 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 follow 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 working standard solution of different concentrations and stored in a deep freezer at -20°C.

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 properly, the mixture was centrifuged at 3000 rpm at about 10 minutes. Then the supernatant used in the TLC method described by [14]. Positive samples were stored at −20°c for HPLC analysis.

High Performance Liquid Chromatography (HPLC)

We optimized an HPLC-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 minutes at 3000rpm in eppendorf tube followed by filtration using 0.2nm MFS filters. The final extracted samples were set to run in the HPLC system described by [15].

Estimation of Hazard Quotient and risk assessment

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

$$\text{Hazard Quotient} = \frac{Estimated\ Daily\ Intake\ (EDI)}{Acceptable\ Daily\ Intake\ (ADI)}$$

The estimated daily intake (EDI) was calculated by the following equation given by Juan et al. (2010).

$$EDI = \frac{(\text{concentration of residue as } \mu g/\ kg) \times (\text{daily intake of food in kg/person})}{\text{Adult body weight (60 kg)}}$$

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 Department of Livestock Services, The per capita availability of milk in Bangladesh was 165.07 ml/day [17]

Acceptable Daily Intake (ADI) is an estimated amount of residue allowed for ingested daily over a lifetime without any appreciable health risk which is expressed on a body weight basis. ADI of amoxicillin and oxytetracycline is 0.2 and 0.03 mg/kg bw/day respectively [18]

A hazard quotient less than or equal to one indicates negligible hazard while greater than one stated the likelihood of harm but it is not the statistical probabilities of occurrence.

Results:

TLC results represented a total prevalence of 7% in raw milk samples and 0% in processed packet milk samples. Screening results for different categories of samples are shown in Table 1. In all positive pooled samples only found the gentamicin residue (4%). Individual samples recognized as positive for all five tested antibiotics in following percentages- Amoxicillin (2%), oxytetracycline (3.3%), streptomycin (1.3%), gentamicin (0.6%) and ceftriaxone (0.6%)

Table 1 **Overall prevalence of antimicrobial residue in milk**

Categories of milk samples	Number of samples tested	Number of positive samples	percentage of positive samples (95% CI)
Pooled samples	50	2	4 (0.5-13.7)
Individual samples	150	12	8 (4-13)
Processed samples	100	-	0 (0.0-3.6)
Total	**300**	**14**	**4.6 (2.5-7.7)**

The results of the UHPLC analysis are presented in Table 2. The oxytetracycline standard concentration was 520 µg/l with the recovery time 6.087 min and peak area of 38953708. The concentrations in five positive samples were found 26.15 µg/l, 6.57 µg/l, 49.73 µg/l, 116 µg/l and 108 µg/l. On the other hand, the value of amoxicillin standard was 200 µg/l and the recovery time 3.7 min with a peak area of 2143200. The three positive samples contained residues as 345 µg/l, 21.5µg/l and 5.85µg/l.

Table 2 Concentration of oxytetracycline and amoxicillin residues in individual milk samples

Antimicrobials	No of positive samples	Maximum conc. (µg/l)	Minimum conc. (µg/l)	Mean conc. (µg/l)[a]	MRL value[b]
Oxytetracycline	5	116	6.57	61.29	100 µg/l
Amoxicillin	3	345	5.85	124	4 µg/l

[a]The average concentration of positive samples

[b]The value of maximum residue limit was collected from Codex report [8]

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 and the results shown in Table 3

Table 3 **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 were 61.29 μg/l and 124 μg/l respectively

[b]Estimated daily intake (EDI) was calculated by the following formula [Milk consumption X mean concentration of residue in milk)/1000]/body weight (60kg)

[c]Acceptable daily intake data derived from the Australian pesticides and veterinary medicines authority [18]

Discussion

We determined the prevalence of antibiotics residue in milk samples and our result was lower in compared to prevalence data found available in different previous literature derived from similar studies. Variation may occur due to the efficiency of the tests used for the screening. We used the TLC method whereas, other studies used microbial inhibition test and commercial kits. Different factors like sample size, location and duration also lead the fluctuation in results. Besides, the use of antimicrobials in dairy cows and found of residues in milk greatly depends on the disease burden of the study area.

We found comparatively higher prevalence of the 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 big volume of pure milk came 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 treated with antibiotics before the period of sample collection. We were able to detect all tested antibiotics - oxytetracycline, amoxicillin, streptomycin, ceftriaxone and gentamicin in individual milk samples but the percentages were lower than the previous findings [19,20] because of the effect of different factors like study area, seasons, disease prevalence in the study area. In pooled samples, only detected gentamicin residue. It might be due to the use of a high dose of gentamicin for treating mastitis in dairy cows in the study areas.

Among processed milk samples none were recognized as positive for selected antibiotics in our study because market milk samples were pasteurized (heat-treated) and few were prepared by ultra-high temperature (UHT) technology. Another important factor was the variation in our packet milk samples including mango milk, chocolate milk, strawberry milk which were supposed to represent the minimum amount of milk. Our result was concordant with an investigation [21] which tested 94 UHT milk and found no samples containing detectable levels of tetracyclines. In the case of pasteurized milk samples, one study in Iran recognized 7.8 % of samples contaminated with oxytetracycline and tetracycline residues but all concentrations were below the MRL [22]. Previous literature also stated that market milk had a lower percentage of antimicrobial residues in comparison to raw milk [6,23].

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 and this finding was supported by previous researcher [24] who also observed amoxicillin residues in raw milk above the MRL but their concentrations was up to 53.7 µg/l which was lower than our result. Besides, we measured the mean value of OTC residue in milk lower than the MRL (l00 µg/1) although two of five positive samples crossed the MRL value. Our mean value was lower than the previous results [25,26] which stated 149.4 µg/l and 150µg/l respectively. Another study carried out in Iran also recognized 218.86 µg/l of OTC residue in milk [27]. Detection of lower concentration may be caused by limited use of OTC in dairy farms at present times.

The Hazard Quotient express the risk posed to human health by consumption of milk having residues and also present the intensity of the hazardous effect. Results revealed the estimated daily intakes (EDI) were much lower than the acceptable daily intakes (ADI) for both amoxicillin and oxytetracycline. 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 found 2 to 100 times lower than the values of the acceptable daily intakes stated by the World Health Organization [25]. Another recent study on milk in Croatia reported the estimated dietary exposure against the detected concentration of amoxicillin, ampicillin, benzylpenicillin, cloxacillin, cephapirin, cefazolin, cefoperazone and ceftiofur were not exceeding the acceptable daily intake [28]. Likewise, daily intake of residues (EDIs) for the average daily milk consumption of 300 ml were 20 to 1640 times lower than the values of acceptable daily intakes (ADIs) based on European Medicines Agency and World Health Organization [29]. A study in India also presented the hazard quotients for oxytetracycline was 0.009 which indicates negligible risk [30]

The time limit of this study was six months only which was very limited to reveal the comprehensive status of different antibiotics residue in various milk samples with associated risk measurement. We have established UHPLC settings for only two veterinary antibiotics - amoxicillin and oxytetracycline residue quantification. To the best of our knowledge, in Bangladesh this study introduced the approach of risk assessment for drug residues present in milk which might be encourage researchers and policymakers to get initiatives on this fact.

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 human health following consumption. By extending this study, develop a database for the concentrations of different antibiotics residue and associated risk levels might facilitate the effort to ensure food safety and public health.

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Conflicts of interest

The authors declare there is no conflict of interest

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