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ORIGINAL ARTICLE

Determination of antibiotic residues in bovine milk by HPLC-DAD and assessment of human health risks in Northwestern Himalayan region, India

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Abstract Antibiotic residues in milk affects economics of dairy industry and poses health risks to consumers. This study aimed to assess health risks associated with presence of antibiotics in 173 raw and pasteurized milk sampled from northwestern Himalayan state of India. The oxytetracycline and amoxicillin were quantitatively analyzed using validated HPLC–DAD. Methods were selective and linear ($R^2 > 0.99$) with decision limit and detection capability of 1.4 and 0.9 µg/kg and 2.5 and 1.5 µg/kg for oxytetracycline and amoxicillin, respectively. Recoveries ranged from 88–98% with relative standard deviation < 10%. Oxytetracycline and amoxicillin were detected in 8.1% and 1.2% samples, with 1.7% and 1.2% samples exceeding the tolerance limits, respectively. Health risk assessment revealed that estimated daily intakes of antibiotics through milk were lower than acceptable daily intakes (ADI). However, children might receive 9–21% of determined ADI through milk consumption only. Therefore, continuous, sub-therapeutic and long term exposures of antibiotics can pose health risk to consumers. Hence, current findings elucidate the need for vigilant monitoring of antibiotics accompanied by educational programs to farmers for adopting good husbandry practices and adherence to withdrawal periods to meet the expectations of food safety and safeguarding human health.

Keywords Antibiotic residues · Milk · HPLC · Health risk · India

Introduction

The increasing human population and changing standards of living escalates the demand for food and other resources. Especially, the demand for safe and wholesome food is ever-increasing and dairy sector has contributed much to the needs of people. Milk is highly consumed commodity and is considered as a nature's perfect food since antiquity. It is an excellent source of nutrients and has an optimal balance of proteins, fats, carbohydrates, vitamins and minerals providing a range of benefits for growth, immunity and development. India is world's largest milk producer with 22 percent of global production. India has also been the leading consumer of dairy products with a sustained growth in the availability of milk and milk products. Milk and milk products also play an important role in boosting food industry and economy with more than 6 billion consumers across the globe (Pereira et al. 2020). According to recent report by IMARC Group, dairy industry in India reached a value of INR 10,540 billion in 2019. However, during recent past, several reports stating contamination of milk with antibiotics and other xenobiotics have challenged its reputation of healthy and nutritious product (Gill et al. 2020; Moudgil et al. 2019).

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In the 1960s dairying constituted only 1% of the Indian agricultural economy, now, the dairy sector is contributing around 26% to total agriculture GDP. However, with the growth and development in dairy sector, prophylactic as well as therapeutic usage of antibiotics has also increased to cope with the increasing demand. Among various antibiotics, tetracyclines' and β-lactams are probably the most widely used class of antibiotics in veterinary medicine for prevention and control of infectious diseases, for growth promotion and for improving production efficiency. However, indiscriminate and improper use of antibiotics as well as poor knowledge of necessary withdrawal time may result in appearance of undesirable antibiotic residues in milk of treated animals. Antibiotic residues in milk from dairy cattle are a priority for milk industry because higher standards of food safety assurance are being required by our society. Milk containing antibiotic residues above tolerance limits, therefore, constitute a violation. The antibiotics in milk not only interfere with fermented milk products processing leading to monetary losses but their long term and continuous exposure also poses a serious health risk to ignorant consumers ranging from allergic reactions, GIT disruptions, carcinogenicity, nephropathy, hepatotoxicity to emergence of bacterial resistance. Nowadays, these antibiotics are also recognized as an emerging environmental problem (O'Connor and Aga 2007). Therefore, analyses of antibiotic residues in food of animal origin is of considerable importance for protection and promotion of 'One Health', and becomes imperative to guarantee food quality and safety.

Antibiotics in milk have become a major consumer concern and there are several international reports of their detection in milk and milk products e.g. from Hungry, Austria, Pakistan, Turkey, China, Greece, Iran and Nepal (Aalipour et al. 2015; Karageorgou et al. 2018; Kaya and Filazi 2010; Khaskheli et al. 2008; Khanal et al. 2018; Rong-wei et al. 2013). But, the information on presence of antibiotic residues in raw and pasteurized milk from India is meagre (Moudgil et al. 2019; Priyanka et al. 2019). According to milk safety and quality survey conducted by FSSAI in India, 1.2% of the milk samples had residues of antibiotics above the permissible limits and oxytetracycline was the main contaminant (FSSAI 2019). However, a survey by Firstmr Business Analytics in India revealed that over 20% milk samples have antibiotic residues, of which around 5% have levels over maximum residue limits (MRLs). Among the antibiotics, amoxicillin was found at higher frequency and at levels higher than acceptable daily intake.

In order to safeguard human health, more stringent policies have been formulated and several regulatory authorities have established MRLs/tolerance limits for veterinary drug residues in food of animal origin (CAC 2018; FSSAI 2018). The Food Safety and Standards Authority of India (FSSAI) has recommended tolerance limit for oxytetracycline as 100 µg/kg in bovine milk (FSSAI 2018). Although, for β-lactam antibiotics such as ampicillin, cloxacillin, FSSAI has set a tolerance limit of 10 µg/kg in milk but amoxicillin in milk is not regulated as per FSSAI till date. However, it has been included in present study because it is regulated by other food safety organizations like US FDA (tolerance level: 10 µg/kg), and is commonly used in food producing animals in India (CDDEP 2016). The Joint FAO/WHO Expert Committee on Food Additives has also recommended an acceptable daily intake (ADI) for oxytetracycline and amoxicillin residues at a concentration of 30 µg/kg bw/day and 2 µg/kg bw/day, respectively (JECFA 2018).

Thus keeping in view, the above mentioned facts, scope of present study was: to validate and optimize analytical methods for detection of oxytetracycline and amoxicillin in bovine milk by high performance liquid chromatography coupled with diode array detector (HPLC–DAD) and conduct cross-sectional study to determine the residues of antibiotics in milk. The selection of antibiotics (oxytetracycline and amoxicillin) was based on their frequent use in veterinary medicine in India (CDDEP 2016; FSSAI 2019; Mutua et al. 2020). Furthermore, potential health risk assessment associated with consumption of milk containing antibiotic residues was also performed. To best of our knowledge, this is the first attempt of such a study being conducted in Himachal Pradesh, a hilly state in northwestern Himalayan regions of India.

Materials and methods

Sampling

A total of one hundred and seventy three bovine milk samples comprising of 128 raw and 45 pasteurized samples were collected randomly from rural areas and retail markets of Himachal Pradesh, India (Longitude: 75° 10′ E; Latitude: 32° 29′ N) during 2018–2019. The samples were subsequently stored at − 20 °C in dark and analyzed within 48 h of collection. Before the analyses, samples were thawed and homogenized in a water bath at 25 oC for 30 min. For method validation and quality control studies, antimicrobial-free milk samples were collected from healthy, untreated cows reared under zero budget natural farming conditions in Northwestern Himalayan Region of India. The blank milk samples were checked for any contamination with targeted antibiotic residues using validated methods.

Reagents and standard solutions

All chemicals of analytical grade were obtained from Central Drug House Ltd., India. Chromatography grade solvents (methanol, acetonitrile) and water were purchased from Avantor perfromance materials, India (RANKEM™). The standards for oxytetracycline and amoxicillin antibiotics (oxytetracycline hydrochloride and amoxicillin trihydrate, Vetranal™) with purities of 96% and 98%, respectively, were procured from Sigma Aldrich, GmBH, Seelze, Germany. Standard stock solutions were prepared by diluting appropriate amount of reference standard in organic solvents (methanol for oxytetracycline and acetonitrile for amoxicillin) to a final concentration of 1 mg/mL. Stock solutions were stored at 4 °C for a maximum of 4 weeks. Working standards solutions for oxytetracycline (25–200 µg/kg) and amoxicillin (2.5–20 µg/kg) were prepared fresh on the day of analysis from stock solutions by appropriate dilution with their respective mobile phases.

Equipment

For detection and quantification of targeted antibiotics in milk, samples were analyzed using Waters® high performance liquid chromatography equipped with a binary pump (Waters, 1525), auto sampler (Waters, 2707), temperature control module for column, and a photo diode array detector (Waters, 2998). The chromatographic separation of targeted analyte was carried out using a Waters® Spherisorb® ODS C-18, (250 × 4.6 mm i.d., 5 µm particle size) column (Waters corporation, USA). Empower® 3 software was used for instrument control and data evaluation.

Hydrophilic-lipophilic balanced (HLB) solid-phase extraction (SPE) cartridge (Waters Oasis® HLB 3 cc/60 mg, Waters Corporation, Milford, Massachusetts, USA) and Rankem PTFE syringe filters (0.22 µm) were used for processing samples. Solid-phase extraction procedures of antibiotics was carried out using a 12 port vacuum manifold with disposable liners (Visiprep™, Sigma Aldrich, USA) coupled with a vacuum pump (ROCKYVAC™, Tarson, India).

Sample preparation

Extraction, clean–up procedures and chromatographic analysis for oxytetracycline was performed following the protocols of Cinquina et al. (2003) and Kumar et al. (2020) with slight modifications. Briefly, 5 mL of milk sample was mixed with 20 mL of 20% trichloroacetic acid. The mixture was then dissolved in 20 mL of 0.1 M Na_2EDTA-Mcllvaine buffer (pH 4.0), vortexed for 2 min followed by sonication for 5 min. Subsequently, the mixture was centrifuged at 4000 × g for 10 min at room temperature. The supernatant was collected and filtered through a fluted Whatman™ filter paper No.1. The filtrate thus collected was applied to SPE cartridge preconditioned with methanol (3 mL) and water (2 mL). After sample loading, the cartridge was washed with 2 mL of 5% methanol and then dried by applying full vacuum for 1 min. Finally, the sample was eluted with 3 mL of methanol. After evaporating the solvent, residues were rE−dissolved in 1 mL of mobile phase, filtered through 0.22 µm PTFE syringe filter and then stored in auto sampler, chromatographic glass vial for further chromatographic analyses.

For analysis of amoxicillin residues in milk matrix, the methods of Martinez-Huelamo et al. (2009) and Camara et al. (2013) with some modifications were followed. Briefly, 5 mL milk and 2 mL acetonitrile were mixed thoroughly to promote protein precipitation. The mixture was vortexed for 1 min and then left to stand for 10 min at room temperature. After centrifugation at 3000 × g for 15 min at 4 °C, supernatant was carefully collected and filtered through a fluted Whatman™ filter paper No.1. Sample extract was then loaded onto HLB cartridge preconditioned sequentially with 3 mL acetonitrile and 3 mL water. The SPE cartridge was then rinsed with 3 mL of water and entire effluent was discarded. The cartridge was then dried (under vacuum) for 3 min. Finally, retained analytes were eluted from cartridge with two volumes of 3 mL of phosphate buffer (pH 3.4) and acetonitrile (30:70, v/v) under gravity. After evaporating the eluent at 40 °C to dryness, residues were reconstituted in 0.5 mL of deionized water, filtered through 0.22 µm syringe filter and then stored in auto sampler LC vial for further chromatographic analysis.

High performance liquid chromatography methods

The chromatographic separation of oxytetracycline residues was carried out using C_{18} column maintained at 25 °C in isocratic conditions with mobile phase: aqueous oxalic acid (0.01 M), acetonitrile and methanol (70:15:15, v/v). The flow rate was fixed at 1.0 ml/min and total run time was kept for 12 min including 1 min of equilibration time. The DAD monitored the eluent at 360 nm and measured spectra from 190 to 400 nm. The sample eluate injection volume was 20 µL.

For detection of amoxicillin, the LC gradient elution was performed using a mobile phase of 25 mM phosphate buffer solution at pH 3.4 (eluent A) and acetonitrile (eluent B) at a flow rate of 1 mL/min. Chromatographic separation was achieved with the following gradient: time (t) = 0–6 min, 90% A and 10% B; 6–12 min, 10–50% B; 12–22 min, 50% B and 50% A; 22–30 min, 5% B and 95% A. The flow rate was 1 mL/min. The DAD monitored the

eluent at 225 nm and measured spectra in UV range (190–400 nm). The sample injection volume was 50 µL.

Method validation and quality control

The methods for extraction, clean-up, detection and quantification of oxytetracycline and amoxicillin residues from milk matrix were optimized and validated in compliance with generally used recommendations and as defined by the European Commission decision 2002/657/EC (EC 2002). The performance parameters evaluated included: linearity, linear range, limit of detection (LOD), limit of quantification (LOQ), sensitivity, trueness, precision, ruggedness and selectivity. For method validation studies, suitable volume of working oxytetracycline and amoxicillin standard solutions were added to 5 mL of blank milk sample.

Linearity was evaluated by calculation of fivE−point linear plots with three replicates based on linear regression and coefficient of determination (R^2). The matrix matched calibrations were performed in the concentration range of 25–200 µg/kg for oxytetracycline and 2.5 to 20 µg/kg for amoxicillin residues. The standard calibration curves were obtained by plotting concentrations (µg/kg) against peak area of analyte.

LOD and LOQ were estimated from calibration curve using the equation given by International Conference on Harmonisation as:

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{\mathrm{m}}, \quad \mathrm{LOQ} = \frac{10 \times \sigma}{\mathrm{m}}$$

where σ = residual standard deviation, and m = slope of calibration curve.

The overall method sensitivity was determined by calculating decision limit (CCα) and detection capability (CCβ) by calibration curve procedure, as described in the directive for analytical method validation procedure 2002/657/EC (EC 2002). Accuracy of method was evaluated by estimating trueness (expressed as recovery percentage) and precision (% relative standard deviation). Recovery experiments, were carried out by spiking blank milk sample with antibiotic standards at five fortification levels in such a way that the levels corresponded to 0.25 × MRL, 0.50 × MRL, 1 × MRL, 1.5 × MRL and 2 × MRL with three replicates for each level i.e. 25, 50, 100, 150 and 200 µg/kg for oxytetracycline and at 2.5, 5, 10, 15 and 20 µg/kg for amoxicillin (MRLs/tolerance limits of 100 µg/kg for oxytetracycline and 10 µg/kg for amoxicillin). After spiking, samples were homogenized by manual shaking and let stand at room temperature for approximately 1 h to allow equilibration of antibiotics with milk matrix before their extraction.

Peak areas of compounds in blank matrix sample spiked before extraction and in the blank matrix sample added just before analysis (matrix matched standards) were used to calculate recovery percentage.

Precision (repeatability) of method is a measure of variability of the results and was determined in terms of relative standard deviation (RSD) of five identical extractions of milk samples spiked with oxytetracycline and amoxicillin standards at same as well as at three different fortification levels. Ruggedness of method was tested by following minor changes in protocol. The selectivity of method was evaluated by analyzing blank milk matrix (n = 10) and mobile phase blanks to determine any interference from endogenous compounds around the retention time window of target analytes.

The oxytetracycline and amoxicillin residues in the milk samples were identified and quantified by comparing the retention time (s) and areas of chromatographic peaks in the sample chromatograms with those of matrix matched antibiotic standard chromatograms. For quality control, each HPLC sequence/batch included: mobile phase, matrix matched standards, test samples, blank and spiked milk samples.

Health risk assessment

The assessment of potential health risks associated with consumption of milk containing antibiotic residues was evaluated by considering the residue analysis results obtained in the present study and per capita availability of milk (kg/day) in Himachal Pradesh, India. Human health risk assessment was performed by calculating estimated daily intakes (EDIs) using HI (Hazard Index) model. The EDI of residues, was calculated for each antibiotic residue using the following equation (Kumar et al. 2018)

$$EDI = \frac{CXF}{W}$$

where C = mean antibiotic residue concentration in milk (µg/kg), F = availability of milk per person in Himachal Pradesh is 0.565 kg/day (NDDB 2019), and, W = mean human body weight (Adult 60 kg and child 15 kg).

EDI was then compared with acceptable daily intake (ADI) established by Joint FAO/WHO Expert Committee on Food Additives (JECFA 2018), for the detected antibiotics to calculate Hazard Index ($HI = EDI/ADI$) and % contribution to ADI as:

$$\%ADI = \frac{100XIntake(\mu g/\mathrm{day})}{ADI(\mu g/\mathrm{kg\ bw/day}) \times \mathrm{body\ weight\ (kg)}}$$

Statistical analysis

All analyses were performed using IBM® SPSS® statistical package (SPSS Inc., Chicago, IL), version 22.0 for windows and Microsoft Excel. Mean, standard error, range, R^2, % RSD etc. were calculated for each targeted analytes using descriptive statistics.

Results and discussion

Method validation

The sample preparation and mobile phase compositions described in previous studies (Cinquina et al. 2003; Martinez-Huelamo et al. 2009; Camara et al. 2013; Kumar et al. 2020) for detection of oxytetracycline and amoxicillin in food matrices were used for optimization of HPLC–DAD conditions in the current investigation. Thus by using the hydrophilic-lipophilic balanced cartridge along with isocratic and gradient conditions and adjusting the flow rate at 1 mL/min for mobile phases resulted in identification of investigated antibiotics with good peak resolutions (Fig. 1, 2 and Online Resource 1). To achieve high sensitivity, maximum absorption wavelength for targeted antibiotics were optimized. Therefore, oxytetracycline and amoxicillin were quantified at detection wavelength of 360 and 225 nm, respectively. Matrix matched calibrations showed that the optimized analytical procedure were linear over the concentration range tested with R^2 value exceeding 0.990 for both oxytetracycline and amoxicillin. LOD values obtained for oxytetracycline and amoxicillin were 2.0 µg/kg and 1.3 µg/kg, respectively. LOQs were also found to be well below the MRLs established by FSSAI and US FDA for targeted antibiotics (Table 1). The present results are comparable with the study conducted by Aalipour et al. (2015) in Iran, where, using an almost similar methodology, detection and quantification limits of 1.18 ng/mL and 4.0 ng/mL, respectively, were obtained for oxytetracycline detection in milk. Similar findings have also been reported from Spain by Camara et al. (2013) wherein, LOD and LOQs were found to be in the range of 2.5–4.0 µg/kg and 3.4–8.6 µg/kg, respectively for detection of different β – lactam antibiotics in milk.

The CCα value was estimated by fortifying three series of blank milk at five concentration levels, and response was plotted against added concentration. The corresponding concentration at y intercept plus 2.33 times the standard deviation (SD) of within-laboratory reproducibility of intercept equals the CCα (Pena et al. 2005). The CCα values obtained were 1.4 and 0.9 µg/kg, for oxytetracycline and amoxicillin, respectively. Furthermore, the corresponding concentration at the decision limit plus 1.64 times the standard deviation of within-laboratory reproducibility of mean measured content at CCα equals the CCβ. The CCβ values obtained for oxytetracycline and amoxicillin were 2.5 and 1.5 µg/kg, respectively.

Antibiotic residues were recovered in the range between 88 and 98% with RSD < 10%. Therefore, recovery values are in accordance with the European commission guidelines, which established a range of 80–120% with RSD < 20% (EC 2002). Analyses of blank samples showed that there were no interference peaks from endogenous compounds present in milk matrix around the retention time window of target analytes. The overall validation data (Table 1) exhibited good results for trueness, repeatability and all other performance parameters for oxytetracycline and amoxicillin in milk demonstrating successful validated methods to carry out routine analysis in accordance to their established tolerance limits.

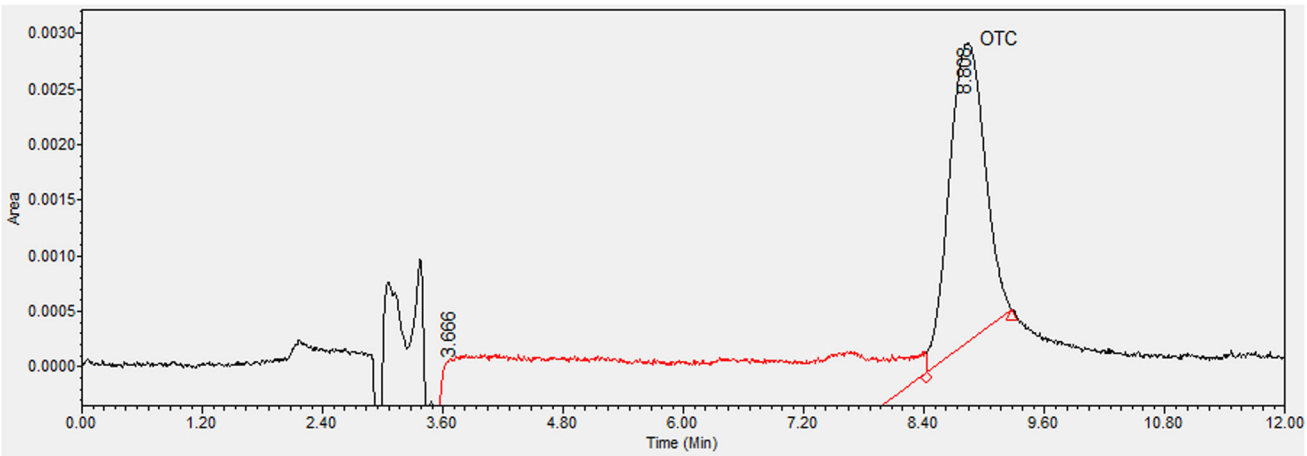

Fig. 1 Representative HPLC-DAD chromatogram of oxytetracycline in milk matrix

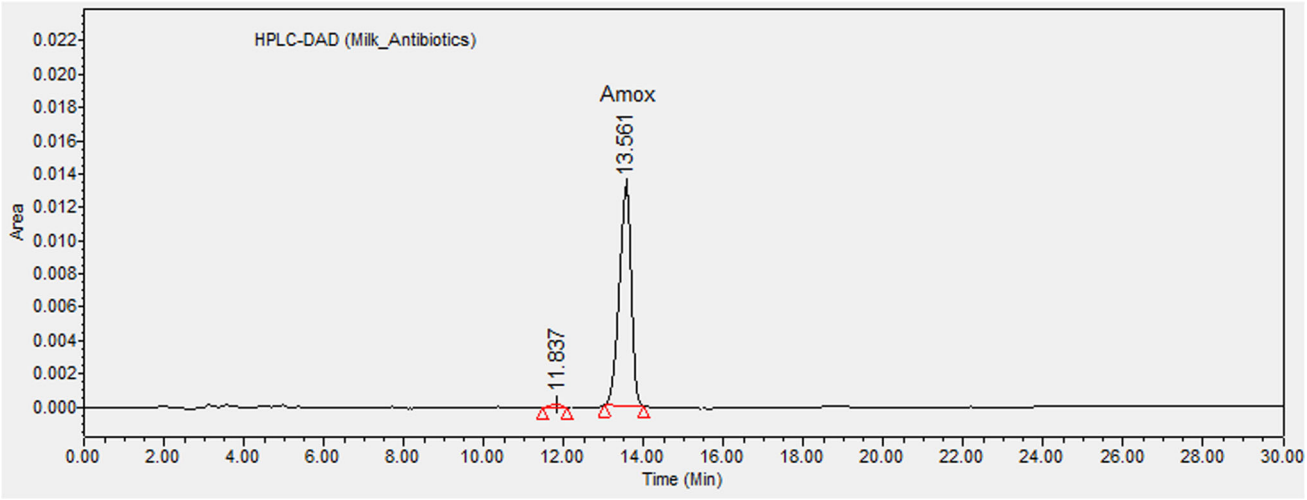

Fig. 2 Representative HPLC-DAD chromatogram of amoxicillin in milk matrix

Table 1 Method validation and quality control parameters for determination of oxytetracycline and amoxicillin antibiotics in milk by HPLC–DAD

Parameters (n = 3)	Oxytetracycline	Amoxicillin
t_R (min)	8.8	13.6
Regression equation (y = mx + c)	y = 1600.1x—2913	y = 2579.8x + 2455.3
Coefficient of determination (R^2)	0.9999	0.9978
LOD (ng/g)	2.0	1.3
LOQ (ng/g)	6.1	3.8
Selectivity	Verified	Verified
Recovery studies		
Recovery ± RSD% at fortification level 1 (0.25 MRL)	96.0 ± 3.6	97.4 ± 4.6
Recovery ± RSD% at fortification level 2 (0.5 MRL)	96.6 ± 2.5	88.6 ± 2.6
Recovery ± RSD% at fortification level 3 (1.0 MRL)	92.2 ± 7.4	90.0 ± 9.8
Recovery ± RSD% at fortification level 4 (1.5 MRL)	91.6 ± 7.2	89.1 ± 14.6
Recovery ± RSD% at fortification level 5 (2 MRL)	92.1 ± 8.9	88.5 ± 6.5
Trueness (overall recovery %)	93.7	90.7
Precision (RSD_{pooled})	5.9	7.6
Method sensitivity	CCα (ng/g)	1.4
	CCβ (ng/g)	2.5
Ruggedness (minor changes)	Verified	Verified

Pooled relative SD (RSD_{pooled}) was calculated as: $RSD_{Pooled} = \sqrt{\frac{RSD_1^2(n_1-1)+RSD_2^2(n_2-1)+\ldots.}{(n_1-1)+(n_2-1)+\ldots.}}$

Occurrence of antibiotics in milk samples

The occurrence of antibiotic residues in milk has become an emerging health issue. In present study, out of a total of 173 milk samples tested, antibiotic residues were detected in 16 (9.2%) samples with mean antibiotic concentration of 60.9 ± 42.8 in positive samples. Out of 16 positive samples, five were found to contain antibiotic residues above their respective tolerance limits.

The detection rate of oxytetracycline in raw (n = 128) and pasteurized (n = 45) milk samples was 9.4% and 4.4%, respectively with concentrations ranging from 31.5–150.6 µg/kg. Three raw milk samples (1.7%) contained oxytetracycline residues above the tolerance limit of 100 µg/kg (Table 2). None of the pasteurized milk sample was found to contain oxytetracycline residues above MRLs. The milk collected from various sources by the milk collection center is pasteurized and sold in retail markets, therefore the dilution of milk could be one of the factor for the less positivity in pasteurized milk samples. However, statistically no significant difference was observed between the positive percentages of raw and

Table 2 Antibiotic residues detected in raw and pasteurized milk samples

Antibiotics	Samples	Positive samples	Mean ± SD[a]	Minimum/maximum quantified (ng/g)	Samples above MRLs
Oxytetracycline	Raw (n = 128)	12/128 (9.4%)	73.1 ± 42.1	34.3/150.6	3/128 (2.3%)
	Pasteurized (n = 45)	2/45 (4.4%)	37.1 ± 7.8	31.5/42.6	-Nil-
	Total	14/173 (8.1%)	67.9 ± 40.9	31.5/150.6	3/173 (1.7%)
Amoxicillin	Raw (n = 128)	2/128 (1.6%)	11.3 ± 1.5	10.2/12.3	2/128 (1.6%)
	Pasteurized (n = 45)	Not detected	–	–	–
	Total	2/173 (1.2%)	11.3 ± 1.5	10.2/12.3	2/173 (1.2%)
Overall	n = 173	16/173 (9.2%)	60.9 ± 42.8	10.2/150.6	5/173 (2.9%)

n, number of samples

[a]Mean ± SD of positive samples

pasteurized milk samples ($p > 0.05$). Considering detection of oxytetracycline in milk, the results obtained were comparable to those reported by other researchers. Sudershan and Bhat (1995) reported the presence of oxytetracycline in 9.0% of market milk samples and 73% of individual animal milk samples from Hyderabad (India). Moudgil et al. (2019) detected oxytetracycline residues in 4.3% of milk samples with 1.65% samples exceeding the MRLs from state of Punjab (India). In a study conducted by Abbasi et al. (2011) to determine the occurrence of antibiotics in milk from Iranian markets, out of 114 samples analyzed, 28.6% and 24.4% of raw and pasteurized milk samples were found positive for oxytetracycline residues. In a similar work by Aalipour et al. (2015), 24% of pasteurized milk samples from Iran contained oxytetracycline residues. However, percentage of positive samples for oxytetracycline residues in the present study was relatively lower than these two studies.

Evaluation of samples for amoxicillin residues revealed that, out of 173 raw and pasteurized milk samples, only 2 raw (1.2%) samples gave positive results with mean concentration of 11.3 ± 1.1 µg/kg. Both samples contained amoxicillin levels above tolerance limit of 10 µg/kg. However, none of the pasteurized milk sample analyzed showed contamination of amoxicillin residues at detectable levels (Table 2). For comparisons of the results obtained for amoxicillin occurrence in milk samples, only a couple of reports are available in published literature regarding the presence of amoxicillin residues in raw and pasteurized milk. The results of current investigation corroborate with the previous study by Priyanka et al. (2019) who reported that 5% of pasteurized milk samples sold in Hisar city contained amoxicillin residues and 3% of total milk samples had levels above MRLs. In another study conducted by Karageorgou et al. (2018), β-lactams were demonstrated as the more frequent antibiotic residues in Greek milk. Similar results has been obtained from earlier studies conducted in Pakistan (Khaskheli et al. 2008) and Turkey (Kaya and Filazi 2010).

Results of current investigation reflects that the levels of oxytetracycline and amoxicillin in pasteurized milk are well below the MRLs and detection capabilities of the method. Although not completely destroyed, but one of the reason for low levels in pasteurized milk could be the poor heat stability of targeted antibiotics (Hassani et al. 2008; Roca et al. 2011). Besides heat stability, other possible reason for low detection percentages might be the stability of antibiotics in pasteurized milk samples stored at different temperatures before reaching the retail markets of hilly Indian state (Rozańska and Osek 2013). However, relatively high detection percentages of antibiotics in raw milk probably be attributed to their frequent use in the treatment of various systemic diseases prevalent in India or the lower LODs of validated analytical procedures (Kumar et al. 2018; Rong-wei et al. 2013). In none of the samples, simultaneous occurrence of more than one type of antibiotic residues was detected. This is in contrary to the findings of Khanal et al. (2018) who reported the occurrence of

more than one type of antibiotic residues in same sample from neighboring country, Nepal.

Excessive use of the antibiotics in lactating animals would result in elevated secretion of their residues in the milk. However, owing to stringent food safety regulations, there have been an increasing research trends with concern about antibiotic residues in milk and their detection accordingly. According to one of the estimates, about 30,000 L of milk are discarded every year due to antibiotic contamination in Europe. Whatever the national and international situations on implementation of food safety regulations are, even the trace presence of such contaminant in food products is alarming and potentially dangerous for consumers' health and therefore needs utmost attentions and risk characterization.

Human health risk assessment of dietary exposure to antibiotics through milk

The results of human health risk assessments are presented in Table 3. The EDIs of detected antibiotics for both adults and children were found to be lower than the ADIs i.e. the hazard index values were less than 1. This indicates that the intake of antibiotic residues does remain clearly below the maximum permissible limits and there is negligible direct risk to health of consumers at current levels of contamination. But, the continuous, sub-therapeutic and long term exposures of antibiotics can be a significant risk to human health. Moreover, considering the concentration of antibiotics detected in positive samples and based on the presented results, estimated daily exposure for children could reach up to about 21% of the defined ADI at short term exposure. While, considering per capita milk consumption in long time, children can be exposed to more possible risk due to their less body weight. Further, it is emphasized that the human health risk assessments did not include the other major food commodities such as eggs, meat, honey etc. Therefore, it is a challenging process to determine the actual dietary consumption of antibiotics through various items of our daily food basket. Hence, any preliminary presumption require an in-depth research and validation and therefore, additional cohort studies involving other food commodities should be performed to ensure overall food safety.

Conclusion

The antibiotic usage in veterinary medicine and failure to follow necessary withdrawal periods may poses a threat to public health especially when the food safety regulations are not strictly enforced. In addition to various adverse health effects that can occur as a result of exposure to veterinary drugs; contributions toward emergence of antibiotic resistance among microorganism is considered as a major future threat to human health. The present study demonstrated that majority of milk produced under virtually natural farming system adopted by the poor and marginal livestock keepers' of Himalayan region in India is free from contaminants. But, a few sample did contained antibiotic residues that too exceeding the safety limits recommended by regulatory authorities. This suggests that withdrawal periods and good husbandry practices are not followed by some uninformed farmers. Although, the estimated daily intake of the consumers was below ADI; however, the low levels of antibiotic residues could still pose adverse health risks. This is being reported probably for the first time from study area and hence, the outcomes of investigation emboldens future monitoring studies with more number of target antibiotics. Therefore, for safeguarding human health it becomes very essential to judiciously use antibiotics in animal husbandry practices,

Table 3 EDI and % ADIs for antibiotic residues found in raw and pasteurized milk samples

Antibiotics	ADI	Age group (weight)	Raw milk			Pasteurized milk		
			EDI	HI	% ADI	EDI	HI	% ADI
Oxytetracycline	30.0	Adult (60 kg)	6.88E−01	2.29E−02	2.29E + 00	3.49E−01	1.16E−02	1.16E + 00
		Children (15 kg)	2.75E + 00	9.18E−02	9.18E + 00	1.40E + 00	4.66E−02	4.66E + 00
Amoxicillin	2.0	Adult (60 kg)	1.06E−01	5.32E−02	5.32E + 00	Not detected		
		Children (15 kg)	4.26E−01	2.13E−01	2.13E + 01			

EDI, Estimated daily intake, *HI*, Hazard index and, *% ADI*, percentage contribution to acceptable daily intake

routinely monitor foods of animal origin for antibiotic residues, and spread awareness among livestock keepers'.

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Author contributions AK, AKP: conceived the study design; AK, NS: performed research and analyzed data; AK, AKP and NS: wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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