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ORIGINAL ARTICLE

# Unveiling antibiotic residue contamination: assessing yak, dzomo, and hill cattle milk from himalayan region through QuEChERS-HPLC approach and health risk assessment

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## Abstract

Milk obtained from Yaks, yak hybrid (Dzomo) and hill cattle (Gauri) is major source of nutrients for people inhabiting Himalayas. In present study, QuEChERS approach together with HPLC-UV was used for detection and quantification of tetracycline residues in 170 raw milk samples of hill cattle (60), dzomo (58) and yaks (52) collected from high altitude regions of Himachal Pradesh, a Western Himalayan state of India. The method validated as per European Commission's guidelines was found to be linear ($R^2 > 0.99$), accurate (recoveries: 80%~90%) precise (RSD ~ 10%) with LOD of 0.55 and 1.37 ng/mL for tetracycline and oxytetracycline, respectively. Antibiotics were detected in 8 samples, with only 3 samples exceeding MRLs. Hazard Index values for exposure to detected antibiotic residues in milk were below 1 suggesting that the population is likely protected from acute health risks associated with such exposure. However, for children, % contribution to acceptable daily intakes of antibiotics only through milk was found to be high indicating probable risks. Therefore, ongoing monitoring and adherence to regulatory standards are important to ensure the safety of milk and other food products. There is also a need to educate farmers and improve their perception about antibiotics and their usage to safeguard consumer's health.

## Highlights

- First study to explore the occurrence of antibiotic residues in yak and yak hybrid milk.
- QuEChERS approach for advancing antibiotic detection is rapid and cost-effective.
- Health risk assessment finds no significant risks to consumers from antibiotic residues in food.

**Keywords** Yak milk · QuEChERS · HPLC-UV · Antibiotics · Health risks · Himalayas

## Introduction

Yaks, their hybrids (Dzomo) and native cow breed (Gauri) are important genetic resources of Himalayan regions. As per 19th livestock census in the state of Himachal Pradesh, the population of yak and indigenous himachali pahari cattle (Gauri) has been estimated to be 1940 and 7, 59,084, respectively (DAH 2023). They provide milk, meat, fur along with draught power to the people inhabiting high altitude regions of Himalayas. Among various animal origin foods, milk is considered an important source of nutrients to humans (Bakaloudi et al. 2020). Therefore, together with nutrition, it is also an integral component of major economies. The milk and milk products obtained from these animals are considered very nutritious and consumed as a staple diet by many tribes. However, factors like harsh climatic conditions, tough terrains, lack of connectivity, poor awareness on food safety issues and limited veterinary health care facilities may contribute towards inadequate animal husbandry practices adopted by the livestock keepers' of high altitude regions (Thakur et al. 2012).

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In animal husbandry practices, antibiotics have been cornerstone for treatment of various ailments and disease prevention. They also played a very significant role in increasing livestock production and their productivity. However, due to their long term application, over the counter sales, self-medication or misuse of antibiotics in animal healthcare, there have been major human health concerns such as emergence of antimicrobial resistance (Rahman et al. 2021). Moreover, and lack of scientific knowledge on withdrawal periods is also leading to occurrence of antibiotic residues in milk. Consumption of such contaminated milk not only poses risk to consumers' health but also has financial implications by interfering with fermentation process owing to starter culture failures. Therefore, if not monitored on routine basis, antibiotic residues in milk can have detrimental effects on human health and economies (Tiseo et al. 2020). To address this issue and safeguard consumers' health, many food safety organizations like USFDA, European Commission, Food Safety and Standards Authority of India (FSSAI) etc. have established maximum residual limits (MRLs) for antibiotics in milk.

For detection and quantification of antibiotics in food matrices, chromatographic and immunological techniques have been used by various researchers (Sharma et al. 2022a). But, the gold standard for measuring antibiotics in food samples is LC-MS/MS. However, HPLC-UV has been found to be more cost effective, requiring less expertise, and is available in much more facilities than LC-MS. Moreover, HPLC-UV has also been found to be very efficient, sensitive, specific and rugged for detection and quantification of antibiotic residues in milk (Kumar et al. 2022). Therefore, for effective determination of tetracycline and oxytetracycline from cattle milk, liquid chromatographs coupled with UV, DAD and MS detectors have been utilized in the past. Most of these currently used analytical methods are based on the pre-treatment procedures such as liquid-liquid extraction (LLE) and solid phase extraction (SPE). But these methods employ large quantities of consumables and are expensive to operate. The use of large amounts of toxic organic solvents can also pose serious environmental concerns. Therefore, quick, easy, cheap, effective, rugged, safe (QuEChERS) method employing dispersive solid-phase extraction (d-SPE) technique for extraction and clean-up of analytes has gained global acceptance as a reliable sample preparation method for residue analyses. This approach was originally developed for extraction of pesticide residues from vegetables but it has now been utilised as a green chemistry approach for detection of antibiotics too (Kumar et al. 2018; Zhang et al. 2019).

Although various studies have been conducted on determination of antibiotics in bovine milk but there are no reports on milk obtained from high altitude regions especially from yaks and dzomo. Therefore, with the use of the QuEChERS approach and HPLC-UV technique, the current study aimed to validate sample preparation and chromatographic technique for detection and quantification of oxytetracycline (OTC) and tetracycline (TTC) residues in yak, dzomo and hill cattle milk obtained from North Western Himalayan region of India and assess human health risks among consumers.

## Materials and methods

### Chemicals and reagents

All the chemical and reagents viz. methanol, acetonitrile, acetone, orthophosphoric acid etc. used in the present study were HPLC grade obtained from Avantor performance materials (RANKEM™) and Merck Life Sciences (EMPLURA®). For QuEChERS protocol, NaCl, $MgSO_4$ and primary secondary amine (PSA) used were procured from Sigma Aldrich (Supelco, Bellefonte, USA). The standards for tetracycline (oxytetracycline, tetracycline) with purities > 97% were procured from Sigma Aldrich (Vetranal™ and Fluka Analytical, GmBH, Germany). Reference standards were initially stored in deep freezer (-20 °C) under dry storage conditions. Deionized water (obtained from Ultra – Q water purification assembly, Jasco Biotech) was used for all the analyses.

For preparing antibiotic stock solutions, reference standards were brought to room temperature and dissolved in methanol, which were stable for 30 days when kept in a dark at 4 °C. Fresh working standard solutions of oxytetracycline and tetracycline (25–500 ng/mL) were prepared by properly mixing, sonicating, and diluting the calculated volumes of standard solution with mobile phase on the day of analysis. For validation studies, multicomponent standard calibration mixture of both oxytetracycline (OTC) and tetracycline (TTC) was utilized to prepare matrix-matched calibration (MMC) standards in blank matrix.

### Instrumentation, preparation of mobile phase and chromatographic conditions

For chromatographic analysis of external standards and antibiotic residues in samples, Shimadzu HPLC coupled with SPD-M20 UV detector, DGU-20 A pump and LC-20AD auto sampler was used. The targeted analytes were separated using Agilent Zorbax Eclipse C-18 RP column (250 mm × 4.6 mm I.D., 5 µm particle size). Shimadzu LC software was used for data assessment and instrument control.

A mixture of 50 mM $KH_2PO_4$, acetonitrile and 0.5 M OPA (80:19.5:0.5, pH 3.5) under isocratic conditions was utilized as mobile phase for chromatographic separation of tetracyclines'. Before injection onto HPLC system, mobile phase was sonicated and filtered through 0.45µ nylon filters in a glass filtration assembly followed by degasification using ultra-sonication for 10 min. The flow rate was kept at 1 mL/min. Total run time was 10 min including 2 min for equilibration. The targeted analytes were detected at a wavelength of 353 nm. The column temperature was kept at 20 °C.

## QuEChERS protocol for sample preparation

Preparation of sample for chromatographic analysis was done using QuEChERS technique with slight modifications (Marinou et al. 2019). Briefly, raw milk sample (1 mL) was vortexed for 1 min. For method validation and quality control studies, an appropriate volume of working multicomponent standard calibration mixture (OTC + TTC) was added to the blank milk sample. For extraction of antibiotics, 1 mL of 1% glacial acetic acid in acetonitrile was added to the homogenized sample followed by vigorous shaking and vortexing for 1 min in a polypropylene tube. The tube was then centrifuged at 10,000 rpm for 5 min at 5 °C in a cooling centrifuge. The sample was then left to stand in the dark for 10 min. The supernatant was then cleaned-up by mixing with QuEChERS salts (150 mg of NaCl, 37.5 mg of $MgSO_4$, and 25 mg of PSA) placed in a polypropylene tube. The tube was hand shaken for 1 min and vortexed for 30 s, and then centrifuged for 5 min at 10,000 rpm at 5 °C. For evaporation and final concentration of sample, 1 mL of clear supernatant was decanted to a clean borosilicate beaker, and allowed to evaporate at 35 °C under vacuum. Finally, the antibiotic residues in beaker were re-dissolved in 2 mL of mobile phase. The eluent (20 µL) was then injected into HPLC-UV system for chromatographic analysis.

## HPLC determination

For chromatographic analyses, current study utilized a C18 analytical column at 20 °C with 50mM $KH_2PO_4$/ MeCN/ OPA (80:19.5:0.5, pH = 3.5) as the mobile phase. The isocratic analysis under the conditions described resulted in sharp, symmetrical peak of OTC and TTC at flow rate of 1.0 mL/min. The mean retention time of OTC and TTC was found to be 5.2 and 6.4 min, respectively. By comparing the retention time and peak area of the sample chromatogram to those of MMC standards performed under the similar operating condition, the analytes was identified and quantified. Each chromatographic sequence included test sample, working standard reference, matrix matched standard (positive control), blank sample (negative control) and mobile phase blank to check for any interference and false positive results.

## Method validation and quality control

The methodology developed in current study was validated following the quality-control regulations mentioned in the European Commission criteria (EC 2002). The performance parameters assessed were: linearity, LOD, LOQ, accuracy, precision, CCα, CCβ, and selectivity.

Since, MRL for tetracyclines' has been established as 100 ng/mL for milk in India by FSSAI (2011), therefore matrix matched calibration curve were plotted at each of the six fortification levels i.e. 0.25 × MRL, 0.5 × MRL, 1 × MRL, 2 × MRL, 4 × MRL, and 5 × MRL with concentrations of 25 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, 400 ng/mL, and 500 ng/mL in triplicates. This allowed for the evaluation of the method's linearity for OTC and TTC. Calibration curve and equations were created by plotting the peak area (Y-axis) against the concentration (X-axis**).**

The limit of detection (LOD) is the lowest concentration of an analyte in a sample that can be detected, but may not always be precisely measured. The limit of quantification (LOQ), in analytical procedures refers to the smallest amount of analyte in a sample that can be precisely and accurately quantified. The LOD and LOQ were estimated from the calibration equation by using following formula in compliance with ICH guidelines (ICH 2005)

$$LOD = \frac{3.3 \times \sigma}{m}$$

$$LOQ = \frac{10 \times \sigma}{m}$$

Where σ: residual standard deviation, m: slope of the calibration curve created for evaluation of method's linearity.

Peak areas of appropriate concentrations of Oxytetracycline and tetracycline standard solutions added to the blank matrix sample before extraction and just before analysis (matrix matched standards were compared for calculation of recovery rate (%) i.e. accuracy. Measure of variability of results is expressed in terms of precision (repeatability), which was was determined by performing the test method on the same batch of samples in the same laboratory in triplicates and outcomes were presented as relative standard deviation (RSD %). Six fortification levels i.e., 25, 50, 100, 200, 400 and 500 ng/mL in triplicate at each fortification level were used to assess the method's overall trueness (accuracy and precision).

The lowest concentration level that has a 1% probability of producing a false positive result is CCα. With a 5% possibility of a false negative conclusion, CCβ is the smallest content detectable. CCα and CCβ were calculated as per EC (2002) using the following equations:

$$CC\alpha = 2.33 \times \frac{\sigma}{S}$$

$$CC\beta = CC\alpha + 1.64 \times \frac{\sigma}{S}$$

Where σ: residual standard deviation, S: slope of the calibration curve.

The method's selectivity was evaluated by analysing blank milk samples ($n = 10$) and mobile phase to determine any interference from endogenous compounds around the retention time window of targeted antibiotic residue.

## Sampling

A total of 170 fresh raw milk samples comprising 60 from hill cattle, 58 from dzomo and 52 from yaks were collected randomly from high altitude regions and pastures of Kinnaur, Lahaul and Spiti districts of Himachal Pradesh, North-Western Himalayan state of India (Fig. 1). The total sample size estimations were based on 12% expected proportion, a 5% margin of error and 95% confidence interval using the formula:

$$n = \frac{1.96^2 Pexp(1 - Pexp)}{d^2}$$

Where, n = sample size; 1.96 = multiplier for 95% confidence interval; $P_{exp}$ = Expected prevalence; d = desired precision / margin of error.

The blank samples (reference) were also collected from apparently healthy animals reared in a Govt. farm under supervision of registered veterinary practitioner with no history of antibiotic treatment for quality control and validation studies. All the samples were labelled and transported to laboratory under ice cold conditions and stored in dark at -20 °C till processing to avoid matrix alteration (curdling and protein precipitation).

## Human health risk assessment

Based on the analytical results obtained in the present study and the intake rate of yak, dzomo and hill cattle milk by the people of study area, the possible health risks associated with consuming milk containing antibiotic residues were calculated. For human health risk assessments, hazard index (HI) model was used based on estimated daily intakes (EDIs) and acceptable daily intakes (ADI). The EDI values for targeted antibiotic residue were determined using the following formula (Sharma et al. 2022b):

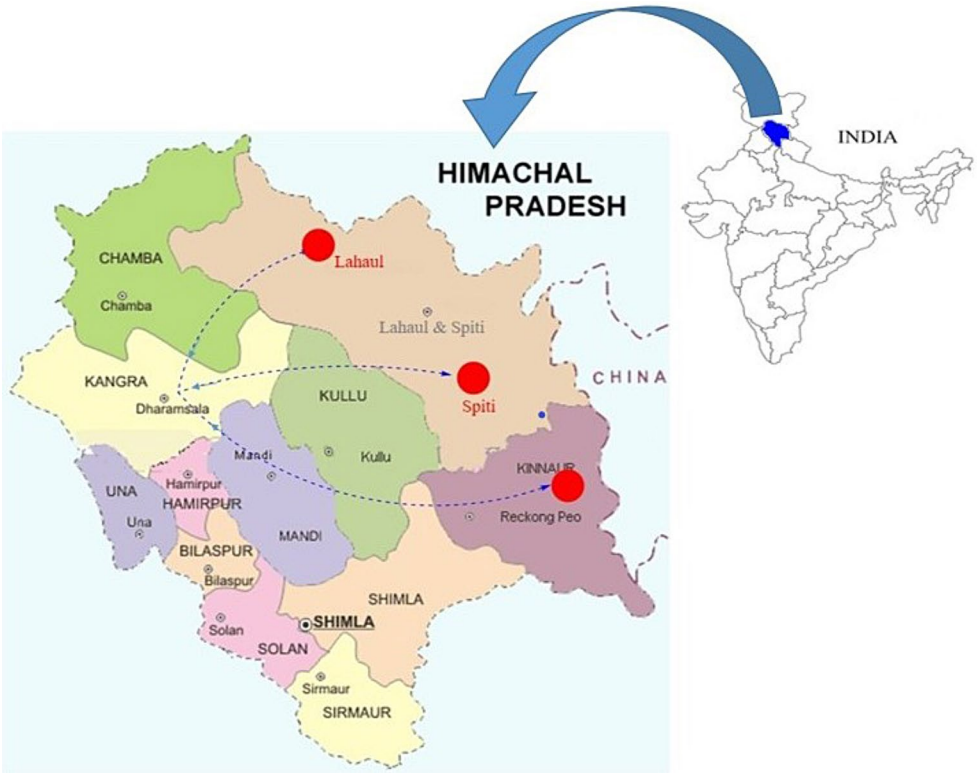


**Fig. 1** Map showing different Himalayan regions of India from where samples were collected

$$EDI = \frac{Ci\ X\ F}{W}$$

Where, Ci = mean of concentration of antibiotic in milk (ng/mL), F = daily dietary intake of milk per person based on report of Department of Animal Husbandry, Govt. of Himachal Pradesh (DAH 2023), W = mean human body weight (60 kg for adults, 15 kg for children).

The HI for the detected antibiotics was then calculated by comparing the EDI is with the acceptable daily intakes (ADIs). The ADI is a precautionary limit set by the world health organization (WHO) based on the lowest "no observed adverse effect level" from a series of toxicological safety assessments. ADI values for antibiotic residues established by WHO (2003) were taken into account while calculating the HI using following equation:

$$Hazard\ Index\ (HI) = \frac{EDI}{ADI}$$

A Hazard Index value below 1 typically indicates that the exposure level is below the threshold of concern for adverse health effects. Therefore, consumer was considered to be adequately protected if HI of a detected antibiotic residue does not exceed unity.

### Statistical analyses

All analyses were performed using IBM® SPSS® statistical package version 22.0 for windows. Mean, standard error, range, $R^2$, RSD % etc. were calculated for each targeted analyte using descriptive statistics.

## Results and discussion

### Method validation

The QuEChERS-HPLC method was validated and used for simultaneous detection of oxytetracycline and tetracycline residues in yak, dzomo and hill cattle milk.

The determination results show that there is a good linear relationship between each concentration and chromatographic response (peak area) in the concentration ranges between 25 ng/mL and 500 ng/mL, with $R^2$ value greater than 0.99 (Table 1).

To verify the accuracy and precision of the method, blank milk samples spiked with OTC and TTC standards at 6 different fortification levels in triplicates were analysed. The results revealed that the recovery rate of the method was 80%~90%, and the relative standard deviation was ~10%. The recovery rates obtained in the present study were similar or even higher than earlier conducted studies on milk matrix. Khosrokhavar et al. (2008) recovered 80 to 97% oxytetracycline residues from spiked milk samples. In another study conducted by Moudgil et al. (2019) from India for detection of tetracyclines in bovine milk, recoveries varied from 83.3 to 111.8% with an RSD between 3.5% and 16.2%. The results of present study are in agreement with a similar study conducted by Akhtar et al. (2021) using QuEChERS protocol for detection of OTC in milk, wherein recoveries of OTC were reported to be varying between 85.67 and 93.53% with RSD of 5.22–15.99%.

The LOD for OTC and TTC were 1.37 and 0.55 ng/mL, respectively (Table 1). Likewise, LOQ were 4.1 and 1.66 ng/mL, respectively. The obtained values of LOD and LOQ were well below the MRLs and therefore method was found to be satisfactory and sensitive for identifying and

**Table 1** Method performance and validation parameters for OTC and TTC residue detection in milk

| Parameter | Oxytetracyline | Tetracycline |
|---|---|---|
| Retention Time (min) | 5.2 ± 0.1 | 6.4 ± 0.1 |
| Regression Equation (y = mx + c) | y = 7.4844x − 17.903 | y = 13.839x − 20.667 |
| Coefficient of determination ($R^2$) | 0.9998 | 0.9999 |
| Linear Range (ng/mL) | 25–500 | 25–500 |
| LOD (ng/mL) | 1.37 | 0.55 |
| LOQ (ng/mL) | 4.1 | 1.66 |
| Recovery Studies | | |
| Recovery ± RSD % at fortification level 1 (0.25 × MRL) | 85.54 ± 10.31 | 82.12 ± 7.06 |
| Recovery ± RSD % at fortification level 2 (0.5 × MRL) | 82.32 ± 8.89 | 87.55 ± 5.24 |
| Recovery ± RSD % at fortification level 3 (1 × MRL) | 84.02 ± 12.03 | 88.12 ± 1.05 |
| Recovery ± RSD % at fortification level 4 (2 × MRL) | 87.7 ± 4.06 | 90.1 ± 2.02 |
| Recovery ± RSD % at fortification level 5 (4 × MRL) | 88.35 ± 6.11 | 86.63 ± 3.96 |
| Recovery ± RSD % at fortification level 6 (5 × MRL) | 90.52 ± 5.10 | 89.15 ± 0.89 |
| Accuracy (Overall recovery % ) | 86.83 ± 3.23 | 87.35 ± 3.37 |
| Precision ($RSD_{Pooled}$ % ) | 3.23 | 3.37 |
| CCα (ng/mL) | 0.96 | 0.39 |
| CCβ (ng/mL) | 1.64 | 0.67 |

measuring OTC and TTC residues in milk. Akhtar et al. (2021) also reported LOD of 0.15 ng/mL for detection of OTC in bovine milk from India using HPLC-DAD method. For quantification of OTC residues in milk, Mamani et al. (2009) and Khosrokhavar et al. (2011) reported LOQ of 51 ng/mL and 60 ng/mL, respectively. However, the results of present study were found to be relatively better.

CCα and CCβ were calculated using the ISO 11,843 approach. CCα for OTC and TTC were 0.96 ng/mL and 0.39 ng/mL, respectively. CCβ for OTC and TTC were 1.64 ng/mL and 0.37 ng/mL, respectively. In a similar study conducted by Kumar et al. (2022), CCα and CCβ values obtained were 1.4 µg/kg and 2.5 µg/kg, respectively for oxytetracycline in bovine milk using HPLC-DAD.

After analyses of blank samples ($n = 10$), no interference peaks were observed in milk matrix around the corresponding retention times of targeted antibiotics (Fig. 2). QuEChERS technique used for detection of antibiotics from yak and dzomo milk was probably employed for the first time in the current study. The results are comparable with earlier conducted investigations by Grabsk et al. (2019), where, they reported extraction of ceftiofur and cloxacillin from dairy milk using the QuEChERS method with an accuracy of 91–99%. The QuEChERS technique was also utilized by Aguilera-Luiz et al. (2008) to extract erythromycin and doxycycline from milk with recoveries ranging from 73.3 to 111.3% and 78.5 to 89.6%, respectively. In present study, the overall validation data (Table 1) exhibited good results for $R^2$, recovery, LOD, LOQ, repeatability and all other performance parameters for extraction and detection of OTC and TTC in milk. For complex matrices like yak, dzomo, and hill cattle milk, the current work thus proposes a highly selective technique. This demonstrated successful validated QuEChERS method and HPLC-UV analysis to carry out chromatographic analysis of real milk samples for OTC and TTC in accordance to their established tolerance limits.

## Detection of antibiotics in raw milk samples

A total of 170 raw milk samples collected directly from animals reared in high altitude regions of Himachal Pradesh, India were analysed; these samples were taken directly from the place of production to the laboratory by refrigerated transport. On the basis of reconnaissance survey conducted in the study region, it was found that tetracyclines' are most commonly used antibiotics in animal husbandry practices, and hence targeted in the current investigation. As shown in Table 2, chromatographic analysis revealed that five samples (2.94%) contained OTC. The absolute mean among positive sample was found to be 242.7 ± 180.4 ng/mL, whereas overall mean of all the samples 7.1 ± 5.7 ng/mL. The positive samples contained OTC residues in the range of 9.03 to 960 ng/mL. The occurrence of OTC in the concentration 960 ng/mL is very alarming indicating failure to follow withdrawal periods by the farmers. Out of 5 positive samples, 2 were found to be containing residue levels above MRL of 100 ng/mL.

For TTC, 3/170 samples (1.76%) were found to be positive with absolute mean of 139.1 ± 95.9 ng/mL and overall mean of 2.4 ± 1.9 ng/mL. Only 1 sample was found to be exceeding level above MRL. The positive samples were containing TTC residues in the range of 39.8–331 ng/mL.

Although, the results of present study are not in agreement with the Kumar et al. (2022) who reported OTC positivity in 8.1% of bovine milk samples from Himachal Pradesh. This may be due to the fact that in current study milk samples were collected from different species distributed in different geographical region. Similarly, Moudgil et al. (2019) also

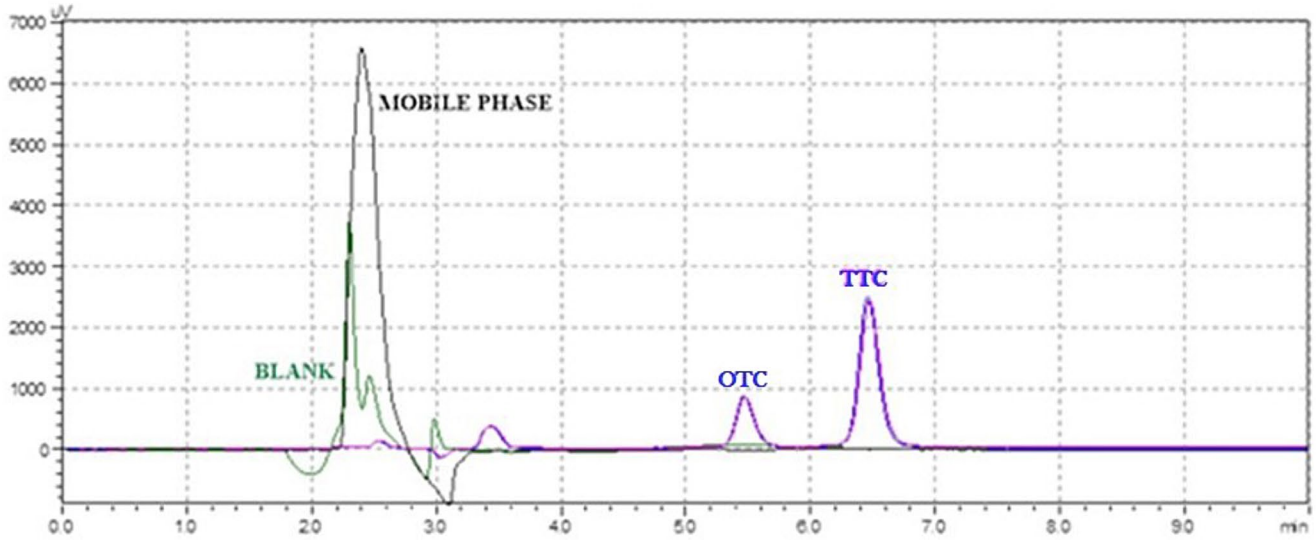


**Fig. 2** Representative overlaid HPLC-UV chromatogram of antibiotic standards, blank milk matrix and mobile phase

**Table 2** Antibiotic residues detected in milk samples ($N = 170$)

| Antibiotics | Positive samples (%) | Absolute mean ± SE * | Overall mean ± SE ** | Min. Quantified (ng/mL) | Max. quantified (ng/mL) | Positive samples above MRL (100 ng/mL) |
|---|---|---|---|---|---|---|
| **OTC** | 5 (2.94%) | 242.7 ± 180.4 | 7.1 ± 5.7 | 9.03 | 960.0 | 2 (1.18%) |
| **TTC** | 3 (1.76%) | 139.1 ± 95.9 | 2.4 ± 1.9 | 39.8 | 331.0 | 1 (0.59%) |
| **Overall** | 8 (4.7%) | | | | | 3 (1.76%) |

*Absolute mean - Mean of positive samples

**Overall mean - Mean of overall samples where the values of negative samples were taken as zero

**Table 3** Frequency of antibiotics detected in hill cattle, dzomo and yak milk

| Sample Type (*n*) | Samples positive | Samples above MRL | Antibiotics detected (frequency) | Source of positive samples (latitude and longitude) |
|---|---|---|---|---|
| **Hill cattle milk (60)** | 3 (5.0%) | 01 (1.7%) | TTC (3) | Kinnaur (31.6096° N, 78.4932° E |
| **Dzomo milk (58)** | 3 (5.2%) | 02 (3.4%) | OTC (3) | Kinnaur (31.5377° N, 78.2754° E) Spiti (32.2411° N, 78.0345° E |
| **Yak milk (52)** | 2 (3.8%) | 00 (0%) | OTC (2) | Kinnaur (31.4255° N, 78.2650° E) |
| **Total (170)** | 08 (4.7%) | 03 (1.76%) | | |

reported 16% (78/492) of dairy farm milk samples positive for antibiotic residues from neighbouring state of Punjab. So, this may also be due to the geographic variations in the sources of samples. In high altitude regions, animal rearing is low budget and milch animals are reared under natural farming conditions primarily for household consumption. This probably acts as major determinant for their overall health and well-being thereby limiting the application of antibiotics. Moreover, it has also been observed that indigenous animals in high altitude regions are resilient to adverse climatic conditions as compared to cross-bred and exotic breeds, making them more disease resistant, requiring relatively less allopathic medicines. People engaged in livestock farming practices in Himalayas are also found to be following ethno-veterinary practices due to poor linkage to the veterinary facilities (Thakur et al. 2024). Whereas, in other parts of the state or country, high yielding milch animals especially crossbreds are reared intensively for production and profits thus making them more prone to infections and diseases. Therefore, frequent usage of antibiotics could be the reason for their higher detection frequency. However, further studies are required to verify this hypothesis.

Species wise analysis of results revealed that milk obtained from dzomo (yak hybrid) and hill cattle showed the highest levels of contamination compared to yaks (Table 3). These results emphasize the importance of monitoring and managing milk quality in diverse livestock populations. Yak milk was least contaminated with antibiotic residues and none was found to have levels above MRL values. This finding is significant as it ensures the safety and quality of the yak milk, meeting consumer expectations for natural and wholesome products. Since, dzomo and hill cattle are also reared for draught purposes i.e. they are often involved in tasks such as plowing, hauling, and transportation in rugged terrains. Therefore, they are predisposed to injuries and thus infections causing relatively frequent administration of antibiotics by the farmers in high altitude regions. This increases the likelihood of antibiotic residues being present in the milk of dzomo and hill cattle. Yaks have very high cultural values are worshipped by Buddhists in high altitude Himalayan regions. Yak milk is considered highly nutritious with numerous health benefits. Therefore, the results of present study signifies the quality of raw yak milk in terms of antibiotic residues.

## Health risk assessment

The cumulative exposure of antibiotic residues though various foods can be very high. Therefore, the monitoring and reporting of antibiotic residues in milk along with the health risk associated becomes very important to protect human health. Based on mean concentration of detected antibiotics in positive milk samples, HI values for adults were calculated to be 2.35E-04, 1.38E-01, and 3.93E-02 for oxytetracycline/tetracycline in yak, dzomo and hill cattle milk, respectively. For child, HI values for oxytetracycline were found to be 9.42E-04, 5.53E-01 in yak and dozomo milk, respectively (Table 4). As evident, Hazard Index (HI) values calculated for both adults and children, based on exposure to detected antibiotic residues through milk consumption, were below 1. As a result, it is presumed that the population is adequately protected from acute health risks associated with such exposure. Similar findings have also been made by Kumar et al. (2022) and Sharma and Kumar (2024) from Himachal Pradesh wherein they reported occurrence of antibiotics in bovine and goat milk but on risk assessments, HI values were found to be less than 1.

**Table 4** Health risk assessment based on Hazard index and % ADI for detected antibiotics in milk

| Sample type | Antibiotic detected | Mean Conc. (ng/mL) | Adult | | | Child | | |
|---|---|---|---|---|---|---|---|---|
| | | | EDI | HI | % ADI | EDI | HI | % ADI |
| Yak milk | OTC | 36.2 | 7.06E-03 | 2.35E-04 | 2.35E-02 | 2.82E-02 | 9.42E-04 | 9.42E-02 |
| Dzomo milk | OTC | 380.4 | 4.15E + 00 | 1.38E-01 | 1.38E + 01 | 1.66E + 01 | 5.53E-01 | 5.53E + 01 |
| Hill Cattle | TTC | 139.1 | 1.18E + 00 | 3.93E-02 | 3.93E + 00 | 4.72E + 00 | 1.57E-01 | 1.57E + 01 |

ADI = Acceptable Daily Intake (30 µg/kg body weight/day); EDI = Estimated Daily intake (µg/kg body weight/day); HI = Hazard Index (EDI/ADI); % ADI = Percent contribution of total dietary intake of milk to ADI i.e. intake as % of ADI

However, the occurrence of OTC and TTC in concentrations of 960 ng/mL and 331 ng/mL is alarming and indicates failure to follow withdrawal periods, which require immediate attention. The similar findings were reported Elizabeta et al. (2011) who estimated the dietary exposure to antibiotic residues detected in milk samples in Macedonia and Croatia. The EDIs were reported to be 2-100 times lower than ADI values. Thus, antibiotics in milk were not considered as a public health issue.

Although, calculated HI values were less than 1, however, % contribution to ADI for oxytetracycline were higher in children. The % contribution to ADI indicates the proportion of the acceptable daily intake of antibiotics that is attributed to milk consumption. A high % contribution suggests that a significant portion of the ADI is being consumed through milk. Children may be more susceptible to the effects of antibiotic residues due to their smaller body size, developing physiology, and potentially higher consumption relative to body weight compared to adults. Moreover, keeping in view the dzomo population and their husbandry practices in high altitude region of Himachal Pradesh together with cumulative exposure of antibiotics through dzomo milk at current level of contamination, children may be at a greater risk than adults.

Since, the current study was limited to only milk analysis in the study area and have not included other food items consumed by the people on daily basis that might be contaminated with antibiotics. Therefore, the present results should be cautiously interpreted considering various reports on antibiotic contamination of different food matrices such as eggs, honey, meat etc.

## Conclusions

Being the first study to explore the occurrence of antibiotic residues in yak and yak hybrid milk, this research addresses an important knowledge gap and contributes to our understanding of antibiotic contamination in milk from unique and culturally significant animal populations. The optimized QuEChERS-HPLC method proved to be very efficient in extracting antibiotics from complex matrix and was fully validated for confirmatory and quantitative purposes. After conducting a comprehensive chromatographic analysis of multiple samples of yak, dzomo and hill cattle milk, it was found that most of the samples are free from tetracyclines' and very few exceeded the MRL values. Moreover, the health risk assessment indicates that the population is adequately protected from acute health risks associated with antibiotic residues in milk. Therefore, this study highlights the significance of providing natural and wholesome food, which is a key expectation among consumers in high altitude regions with limited infrastructure and healthcare facilities and especially those seeking healthier and more sustainable food options. Measures such as stricter regulations on antibiotic use in dairy farming, enhanced monitoring of milk quality, education on safe milk consumption practices, and dietary diversification may be warranted to address food safety concerns. Future research could explore integrating data from multiple food sources, wide range of antibiotics, seasonal variations in antibiotic presence or investigate the effectiveness of antibiotic monitoring programs in animal farming communities of Himalyan regions.

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**Code availability** Not applicable.

## Declarations

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

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

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