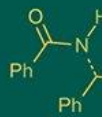


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Occurrence of oxytetracycline residues in milk and dahi from Hisar, Haryana

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Abstract

Oxytetracycline residues in milk and fermented dairy products are of growing concern due to their potential impact on consumer health and regulatory compliance. The present study aimed to determine the occurrence of oxytetracycline residues in milk and *dahi* samples collected from Hisar, Haryana, using an optimized HPLC method. Chromatographic separation was achieved on a C18 column with a mobile phase consisting of 0.01 M oxalic acid and a methanol: acetonitrile mixture (40:60) in a 65:35 ratio under isocratic conditions. Detection was carried out at 355 nm with a flow rate of 0.75 mL/min and a total analytical runtime of 8 minutes. A total of 100 milk and 100 *dahi* samples were analysed. The results revealed that 79% of milk samples and 87% of *dahi* samples were free from detectable oxytetracycline residues. However, 9% of milk and 5% of *dahi* samples contained residues below the maximum residue limit (MRL), while 12% of milk and 8% of *dahi* samples exceeded the established MRL. Statistically, the findings indicate a higher incidence of residue contamination in milk compared to *dahi*, possibly due to processing effects such as fermentation and microbial degradation. The presence of oxytetracycline residues above permissible limits in a notable proportion of samples highlights the urgent need for strict monitoring and regulation of antibiotic usage in dairy animals. Regular surveillance and adherence to withdrawal periods are essential to ensure food safety, mitigate antibiotic resistance, and protect public health.

Keywords: Oxytetracycline, Milk, *Dahi*, HPLC, Antibiotic residues, Maximum residue limit.

1. Introduction

India is the largest producer of milk globally (NDDB, 2023); however, it does not rank among the leading exporters of dairy products, primarily due to challenges in meeting international food safety and quality standards. One of the key factors contributing to this limitation is the presence of chemical contaminants in milk and dairy products, including pesticides, heavy metals, and antibiotic residues (Moudgil *et al.*, 2019) ^[14]. While global trade platforms demand zero or minimal chemical presence, domestic supply chains often grapple with containment. While pesticides and heavy metals generally enter milk through environmental exposure, and contaminated feed antibiotic residues originate directly from the systematic use of veterinary drugs in dairy animals. Antibiotic residues are defined as pharmacologically active substances, including their parent compounds and metabolites that persist in food products derived from animals treated with veterinary drugs (EC, 2010). The indiscriminate and unregulated use of antibiotics in livestock for therapeutic, prophylactic, and growth-promoting purposes has led to the widespread occurrence of these residues in milk. Such residues pose significant risks to consumer health, including hypersensitivity reactions, carcinogenic and mutagenic effects, teratogenicity, nephrotoxicity, and disruption of the normal intestinal microbiota (Priyanka *et al.*, 2017) ^[17]. Crucially, the chronic ingestion of sub-lethal antibiotic doses via daily dairy consumption is a primary driver behind the global emergence of multi-drug resistant (MDR) bacterial strains. Additionally, the presence of antibiotics in milk can adversely affect dairy processing by inhibiting starter cultures, leading to fermentation failures and reduced product quality in fermented commodities (Kebede *et al.*, 2014) ^[9]. Among the various classes of antibiotics used in veterinary practice, tetracyclines, sulphonamides, and fluoroquinolones are commonly employed due to their broad-spectrum activity and cost-effectiveness. Tetracyclines,

particularly oxytetracycline, are extensively used because of their easy availability, low cost, and effectiveness against a wide range of Gram-positive and Gram-negative bacterial infections, such as mastitis and respiratory tract infections (Jank *et al.*, 2017; Martins *et al.*, 2015) [8, 13]. However, improper adherence to withdrawal periods, lack of awareness among farmers, over-the-counter availability without veterinary prescriptions, and insufficient regulatory enforcement contribute to the persistence of these residues in milk and milk products (Andrew *et al.*, 2009; Beyene, 2015; Sachi *et al.*, 2019) [2, 3, 19].

To safeguard consumer health, regulatory authorities have established Maximum Residue Limits (MRLs) for antibiotics in foods of animal origin. The Maximum Residue Limit represents the highest concentration of a residue legally permitted in food. In India, the Food Safety and Standards Authority of India (FSSAI) has set an MRL of 100 µg/kg for tetracyclines in milk and dairy products, aligning with Codex Alimentarius standards (CAC, 2017; FSSAI, 2019). Despite regulatory measures, surveillance surveys have indicated that antibiotic residues continue to be detected in milk samples, with a proportion exceeding permissible limits, highlighting ongoing concerns regarding food safety and quality. FSSAI has conducted a nationwide survey which showed that while milk is largely safe with respect to chemical adulterants, contamination due to aflatoxin M1 and antibiotic residues is more serious problem than adulteration and the quality concerns persist. The survey further showed that 77 samples (out of 6,432 samples) had antibiotics residues above the permissible limits (FSSAI, 2019).

The detection of antibiotic residues in dairy products requires reliable and sensitive analytical techniques. Rapid screening methods such as microbiological assays, immunoassays, and biosensors are commonly used for preliminary detection; however, these methods lack specificity, are prone to false positives, and cannot differentiate between individual antibiotics within the same class (Liu *et al.*, 2009; Pikkemaat, 2009; Raz *et al.*, 2009) [12, 16, 18]. In contrast, chromatographic techniques, particularly high-performance liquid chromatography (HPLC) coupled with UV-Vis or Photodiode Array (PDA) detection, offer superior sensitivity, selectivity, and accuracy for the identification and quantification of specific antibiotic residues in complex food matrices.

Although several studies have reported the presence of antibiotic residues in milk, limited information is available regarding their occurrence in fermented dairy products such as *dahi*, where processing conditions like fermentation, heat treatment, and bacterial metabolism may influence residue levels. Therefore, there is a need to systematically investigate the occurrence of specific antibiotic residues in both raw and processed dairy products to map their fate along the processing chain.

In this context, the present study was undertaken to evaluate the occurrence of oxytetracycline residues in milk and *dahi* samples collected from Hisar, Haryana, using a validated HPLC method. The study aims to generate data on the prevalence of oxytetracycline contamination and assess

compliance with established regulatory limits, thereby contributing to food safety monitoring and public health protection within the region.

2 Materials and methods

2.1 Sample collection

A total of 200 samples comprising 100 raw milk and 100 *dahi* samples were collected from different locations in Hisar, Haryana, India. The samples represented both organized (dairy plants and commercial booths) and unorganized (local vendors and direct farm gates) sectors to ensure an unbiased assessment of the market distribution. All samples were collected in sterile containers, transported to the laboratory under refrigerated conditions, and stored at 4 °C until further analysis. The analysis was carried out within a stipulated time period to prevent any potential climate-induced or to avoid any degradation of residues.

2.2 Chemicals and reagents

The analytical standard of oxytetracycline (purity >98%) was procured from Sigma-Aldrich. HPLC-grade methanol and acetonitrile, along with analytical grade oxalic acid (purity >99.5%), were obtained from Loba Chemie Pvt. Ltd. HPLC-grade water was prepared in the laboratory using a Millipore Synergy® water purification system with a resistivity of 18.2 MΩ·cm. All chemical reagents were filtered through a 0.45 µm membrane filter and degassed ultrasonically prior to use.

2.3 Preparation of reagents and standard solutions

The mobile phase consisted of solvent A (0.01 M oxalic acid) and solvent B (methanol, 40:60, v/v), mixed in a ratio of 65:35 as detailed in Table 1. Solvent A was prepared by dissolving precisely weighed oxalic acid in HPLC-grade water, while solvent B was prepared by mixing methanol and acetonitrile in the specified ratio.

A stock solution of oxytetracycline (100 µg/mL) was prepared by dissolving 1 mg of oxytetracycline standard in methanol and making up the volume to 10 mL in a Class A volumetric flask. The stock solution was wrapped in aluminium foil to protect it from photo-degradation and stored at -20 °C until use. Working standard solutions were prepared by appropriate dilution of the stock solution with methanol to obtain concentrations in the required analytical range.

2.4 Sample preparation and extraction

Milk and *dahi* samples were subjected to extraction procedure as reported by Kurjogi *et al.* (2019) with some modifications. The procedure used for extraction of oxytetracycline from milk and *dahi* is shown in Fig. 1 and is as follows. For milk, 10 mL of sample was taken in a centrifuge tube, followed by vortexing for 1 min. Subsequently, 1 mL trifluoroacetic acid (TFA) was added as a protein-precipitating agent and mixed thoroughly. The mixture was centrifuged at 1782 x g for 15 min, and the clear supernatant was collected. The extract was filtered through a 0.22 µm syringe filter prior to HPLC analysis.

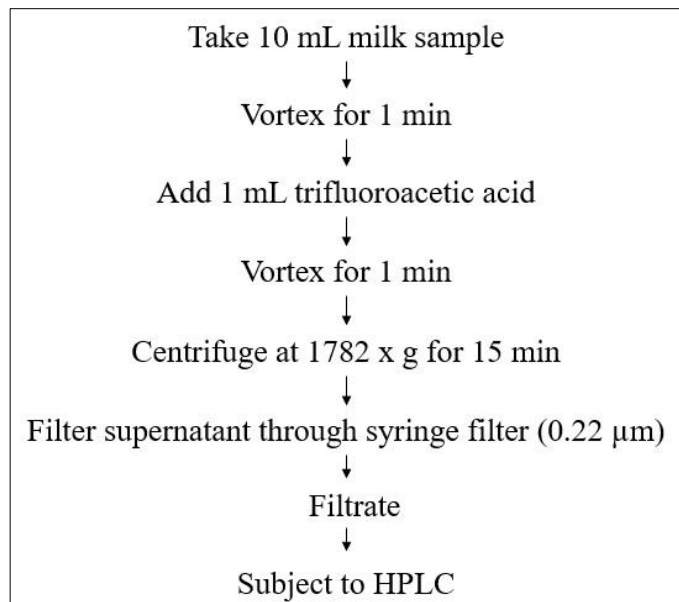


Fig 1: Schematic representation of procedure for extraction of oxytetracycline from Milk

For *dahi*, 5 g of sample was taken in a centrifuge tube, followed by the addition of 5 mL of HPLC-grade water and vortexing for 1 min to ensure complete homogenization of the curd matrix. Afterwards, the procedure (TFA addition, centrifugation, and filtration) was carried out identically to that of milk.

2.5 HPLC instrumentation and conditions

Chromatographic analysis was performed using a Shimadzu HPLC system equipped with a DGU-20A3 degasser, LC-20AD pumps, and an SPD-20A UV–Vis detector. Separation was achieved on a C18 column (4.6 mm × 150 mm, 5 μm particle size). The mobile phase consisted of 0.01 M oxalic acid and methanol (40:60) in a ratio of 65:35, delivered in isocratic mode at a flow rate of 0.75 mL/min. The comprehensive chromatographic conditions optimized for resolution and baseline stability are summarized in Table 1.

Table 1: Chromatographic conditions for detection of oxytetracycline in milk and *dahi*

Parameters	Conditions
Analyte	Oxytetracycline
Column	C18
Solvent A	0.01M oxalic acid
Solvent B	Methanol: Acetonitrile (40:60)
Ratio of Solvent A:B	65:35
Detection wavelength	355 nm
Flow rate	0.75 mL/min.
Flow type	Isocratic
Oven temperature	35 °C
Injection volume	20 μl
Runtime	8 min

Detection was carried out at 355 nm, with an injection volume of 20 μL and a total runtime of 8 min. The column oven temperature was maintained at 35 °C. Data acquisition and processing were performed using LabSolutions software. Peak identification was established based on the retention time of the standard, as illustrated in the representative chromatograms (Fig. 2).

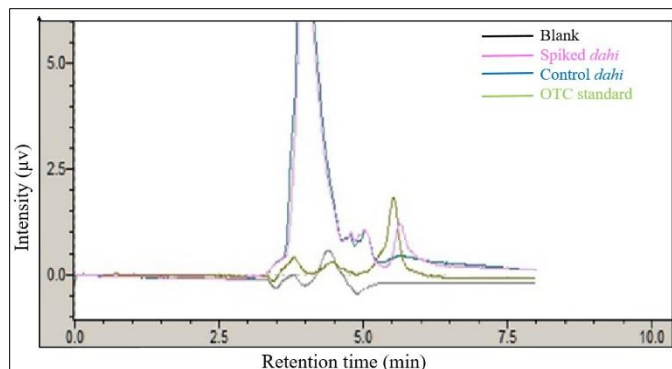


Fig. 2: Representative HPLC chromatograms of oxytetracycline standards, solvent blank (methanol), control *dahi* sample, and spiked *dahi* samples

2.6 Statistical analysis

The data obtained from the analysis were subjected to statistical evaluation using SPSS software. Results were expressed as mean ± standard deviation (SD) to represent the central tendency and variability of the measurements. Where applicable, statistical significance between groups (milk vs. *dahi* contamination rates) was determined at a 1% level of significance ($p < 0.01$).

3 Results and Discussion

3.1 Occurrence of oxytetracycline residues in milk and dahi

The occurrence of oxytetracycline residues in milk and *dahi* samples collected from Hisar, Haryana is presented in Table 2.

Table 2: Occurrence of oxytetracycline residues in milk and *dahi*

Level of oxytetracycline	Milk	<i>Dahi</i>
Not detected	79	87
Below MRL	9	5
Above MRL	12	8
Total	100	100

Out of the total 100 milk samples analysed, 79% were found to be free from detectable residues, whereas 9% contained oxytetracycline below the maximum residue limit (MRL) of 100 μg/kg. However, 12% of the samples exceeded the prescribed MRL. In the case of *dahi*, 87% of samples were free from detectable residues, 5% contained residues below the MRL, and 8% exceeded the permissible limit. Similar findings were also reported by Chauhan *et al.* (2019) [4], in a study conducted for determination of tetracycline residues in milk by HPLC in Hisar city, 6% samples of milk were found to be contaminated with oxytetracycline and 3% with tetracycline.

The results clearly indicate a higher prevalence of oxytetracycline contamination in milk as compared to *dahi*. This difference may be attributed to the processing conditions involved in *dahi* preparation, particularly fermentation, which could contribute to the partial degradation or reduction of antibiotic residues. During fermentation, the metabolic activity of lactic acid bacteria (LAB) such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus* can facilitate chemical transformations or binding of the antibiotic molecules. Similar observations have been reported in previous studies, where fermentation and microbial activity were found to influence the stability of certain antibiotic compounds. In addition to microbial-

mediated effects, thermal processing has also been reported to influence the stability of tetracycline residues in milk. The findings of the present study are in agreement with those reported by Kellnerová *et al.* (2015) ^[10], who investigated the effect of pasteurization on tetracycline residues and observed a reduction of 5.74% in tetracycline and 15.3% in oxytetracycline following high-temperature pasteurization (85 °C for 3 s). This further supports the notion that both thermal treatment and fermentation processes play a significant role in reducing antibiotic residue levels in dairy products.

3.2 Concentration levels of oxytetracycline

The concentration of oxytetracycline in positive samples varied within a wide range reflecting erratic dosing habits. Samples containing residues below the MRL indicated low-level exposure, possibly due to partial metabolism of the drug or adherence to withdrawal periods to some extent. However, the presence of residues exceeding the MRL in a significant proportion of samples suggests improper or indiscriminate use of antibiotics in dairy animals without professional veterinary oversight.

The higher percentage of samples exceeding MRL in milk (12%) compared to *dahi* (8%) further supports the hypothesis that processing conditions may reduce residue levels. Furthermore, the thermal processing (boiling or pasteurization) that milk undergoes prior to *dahi* inoculation might contribute to the thermal cleavage of the tetracycline ring structure. Nevertheless, the persistence of oxytetracycline in both matrices highlights its stability and resistance to complete degradation under normal processing conditions.

3.3 Comparison with previous studies

The findings of the present study are in agreement with earlier reports indicating the widespread occurrence of antibiotic residues in milk in different regions of India. Several studies have reported varying levels of tetracycline residues, with a proportion of samples exceeding the permissible limits. The variation in residue levels across studies may be attributed to differences in animal management practices, antibiotic usage patterns, and regulatory enforcement.

Studies on fermented dairy products have demonstrated a reduction in antibiotic residues during fermentation, with the extent of reduction influenced by factors such as pH, microbial strain activity, initial residue concentration, and fermentation duration. The comparatively lower incidence of residues in *dahi* observed in the present study aligns with these findings, suggesting that lactic fermentation acts as a partial bioremediation barrier. These results are in agreement with Ahmad *et al.* (2021) ^[1], who reported that microorganisms can facilitate the biodegradation of tetracyclines through structural modifications, including opening of the ring structure and cleavage of functional groups.

3.4 Implications for public health and dairy processing

The presence of oxytetracycline residues above MRL in both milk and *dahi* is a matter of concern from both public health and technological perspectives. Consumption of such contaminated products may lead to adverse health effects, including antibiotic resistance, allergic reactions, and disruption of gut microbiota. The transmission of residue-

laden dairy products to infants and vulnerable populations can complicate pediatric clinical treatments due to localized resistance build-up. Furthermore, antibiotic residues can interfere with dairy processing by inhibiting starter cultures, leading to poor fermentation insufficient curd setting, curd whey-off, and reduced product quality. This economic impact is substantial for commercial dairies experiencing production batch failures.

The results emphasize the need for strict adherence to withdrawal periods following antibiotic treatment in dairy animals. Inadequate knowledge among farmers and lack of regulatory enforcement are major contributors to the persistence of residues in milk.

3.5 Statistical analysis

Statistical analysis of the data revealed significant differences ($p < 0.01$) between the proportion of contaminated and non-contaminated samples. The mean concentration of oxytetracycline in positive samples showed variability, indicating inconsistent antibiotic usage practices among different farms. The standard deviation values further reflect the heterogeneity in residue levels among different samples, validating that risk distribution is dependent on individual farm management rather than a uniform regional trend.

Overall, the results highlight the continued presence of oxytetracycline residues in dairy products and underscore the need for improved monitoring, awareness, and regulatory control to ensure food safety.

4 Conclusion

The present study provides important insights into the occurrence of oxytetracycline residues in milk and *dahi* samples collected from Hisar, Haryana. The findings revealed that while a majority of the samples were free from detectable residues, a notable proportion contained oxytetracycline, including levels exceeding the prescribed maximum residue limit (MRL). The higher incidence of contamination observed in milk compared to *dahi* suggests that processing conditions such as fermentation and preparatory heat treatment may contribute to a partial reduction of antibiotic residues. The detection of residues above permissible limits in both milk and *dahi* raises significant concerns regarding the indiscriminate use of antibiotics in dairy animals and non-adherence to recommended withdrawal periods. Such practices not only pose potential health risks to consumers but also compromise the safety and quality of dairy products.

Overall, the study underscores the need for continuous monitoring and stringent regulatory control of antibiotic usage in the dairy sector. Increasing awareness among farmers, regarding withdrawal periods, promoting judicious use of veterinary drugs, mandating prescription-only sales, and strengthening surveillance systems are essential steps to minimize residue contamination and ensure compliance with food safety standards.

Conflict of Interest

The authors declare no conflict of interest.

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