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Screening for Antibiotic Residues in Milk and Study of Antibiotic Resistance of Isolates from Milk

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Abstract

Antibiotics are widely employed in veterinary practice, particularly for the treatment of mastitis and dry cow therapy. Such practices may lead to the secretion of antibiotic residues in milk, leading to public health concerns. The present study aimed to detect residues of antibiotics in milk samples obtained from different milk-producing animals, including native and hybrid cows, buffalo, sheep, and goat and to evaluate antibiotic resistance patterns in bacteria recovered from these samples. Five milk samples were analysed using the five-plate screening method and thin-layer chromatography for antibiotic residues detection. Milk samples collected from hybrid cows and sheep showed detectable levels of antibiotic residues. Bacterial isolates obtained from the milk samples were further screened for antibiotic resistance. The isolate from native cow milk exhibited resistance to methicillin, while isolates from hybrid cow and buffalo milk showed resistance to erythromycin. The resistance profiles observed in the study suggest the emergence of antimicrobial resistance associated with continuous or repeated antibiotic exposure during therapeutic applications. Consumer safety in ensured by regulatory authorities by establishing maximum residue limits (MRLs) for antibiotic residues found in food products. Therefore, routine monitoring by food testing laboratories is essential to make sure that levels of antibiotic residue in milk remain less than the prescribed MRLs and to minimize the risk associated with antimicrobial resistance.

Keywords: Antibiotic residues, Milk, Antibiotic resistance, Veterinary drugs, Maximum Residue Limit (MRL).

Introduction

Milk is a nutritionally rich food containing proteins, fats, vitamins, minerals, and essential amino acids, but it is also highly susceptible to contamination by chemical and microbiological hazards. Among these, antibiotic residues and antibiotic-resistant bacteria pose significant public health concerns. The widespread use of antibiotics in dairy animals for therapeutic, prophylactic and growth-promoting purposes - particularly in developing countries like India—has contributed to the presence of drug residues in milk and the emergence of antimicrobial resistance[1,2].

Common veterinary antibiotics include tetracyclines, β-lactams, sulfonamides, fluoroquinolones, macrolides, aminoglycosides, and cephalosporins. Their misuse, especially failure to observe withdrawal periods, leads to the persistence of residues in milk in various forms (parent compounds, metabolites, or conjugates). Consumption of contaminated milk may result in allergic reactions, toxicity, disruption of gut microbiota, and the development of antimicrobial resistance[1]. Furthermore, a substantial proportion of administered antibiotics (30–70%) is excreted unchanged into the environment, contributing to environmental contamination and resistance selection[2,3].

Antibiotics are extensively used in dairy farming, particularly in mastitis management and dry cow therapy, making these practices major sources of residues in milk. β-lactams (e.g., ampicillin, amoxicillin, penicillin G, cephalosporins) are among the most frequently detected residues due to their widespread use[4], while macrolides such as erythromycin and tylosin are also commonly employed. Improper use, including sub-therapeutic dosing, not only results in residue persistence but also promotes the selection of resistant bacterial strains.

Exposure to antibiotic residues has been associated with adverse health effects, including carcinogenicity, mutagenicity, teratogenicity, hepatotoxicity, nephrotoxicity, and allergic reactions[5,6]. Additionally, residues may persist through processing and concentrate in dairy products such as milk powder and ice cream, posing further food safety concerns. While heat treatments like pasteurization reduce microbial load, they do not reliably eliminate all antibiotic residues.

Regulatory agencies such as Codex Alimentarius, USFDA, EFSA, and NAFDAC have established maximum residue limits (MRLs) to ensure food safety[7].

However, residues exceeding permissible levels are still reported, largely due to poor compliance, lack of awareness among farmers, and improper treatment practices[8]. Adherence to drug-specific withdrawal periods—typically ranging from 3 to 6 days or longer—is critical to prevent residue contamination[9,10].

In parallel, milk serves as a reservoir for antibiotic-resistant bacteria, which can enter the human food chain through raw or inadequately processed dairy products. These bacteria may transfer resistance genes to human pathogens, compromising the effectiveness of clinically important antibiotics[1,2]. Reports indicate that bacterial isolates from milk frequently exhibit resistance to commonly used antimicrobials, reflecting the widespread and often indiscriminate use of antibiotics in livestock. Therefore, simultaneous monitoring of antibiotic residues and assessment of antimicrobial resistance in milk are essential for ensuring food safety and protecting public health. Detection methods such as microbiological inhibition assays, immunoassays, and chromatographic techniques are widely used, alongside antimicrobial susceptibility testing methods including disk diffusion and minimum inhibitory concentration (MIC) assays. In this context, the present study aims to detect antibiotic residues in milk samples and evaluate antibiotic resistance patterns among bacterial isolates, contributing to improved milk quality monitoring and mitigation of antimicrobial resistance risks.

Materials and Methods

Sample collection: Milk samples were collected from different mammals, such as hybrid cow, native cow, buffalo, goat and sheep from regions of Chikkaballapura, Srinivasapura (Karnataka) and Hosur (Tamil Nadu). This was then stored at 4°C before analysis.

Five plate screening test: The five-plate screening test, a microbiological agar diffusion method used to detect antibiotic residues in foods of animal origin, was used to screen milk samples[11]. Two culture media were employed: Mueller-Hinton Agar (MHA) and test agar (TA). Four bacterial test organisms—*Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus mutans*—were swabbed onto each plate. Five wells were created in each agar plate using a cork borer, and 10 µL of each milk sample was dispensed into the wells. The plates were then incubated at 37°C for 24–48 hours. After incubation, the plates were examined for zones of inhibition around the wells, indicating the presence of antibacterial substances, if any.

Thin layer chromatography: The coupling of thin-layer chromatography (TLC) with microbiological detection has been widely used for the identification and quantification of various antibiotics. This approach is considered simple, cost-effective, and relatively sensitive and specific. The application of bioautography combined with TLC for detecting antibiotic residues in milk has been previously demonstrated[12,13].

The antibiotic standards used in this study included clavulanate, tetracycline, amoxycillin, ciprofloxacin, and doxycycline. The TLC plate was marked into 10 channels, of which the first five were designated for milk samples and the remaining five for antibiotic standards. Onto the marked channels, 10–15 µL of each milk sample and standard solution was applied.

The plate was allowed to air-dry for a few minutes and then placed in a TLC chamber containing the mobile phase (methanol and acetone in a 1:1 ratio). The chamber was left undisturbed for 1–2 hours to allow solvent migration. Once the solvent front reached approximately three-fourths of the plate, it was removed, air-dried, and visualized under UV light to observe the separated spots.

Isolation of bacteria from milk samples: Serially diluted samples (10^{-3}, 10^{-4}) was spread evenly over the surface of nutrient agar plates using a sterile glass rod and incubated at 37°C for 48 hours. Gram staining was done to identify the isolated colonies.

Determination of Antibiotic Resistance of Bacterial Isolates: The bacterial isolates were subcultured in nutrient broth and incubated to obtain actively growing cultures. The d is k diffusion technique was used to study antibiotic resistance of the isolates obtained. The cultures were uniformly swabbed onto the surface of MHA plates using sterile cotton swabs. Five antibiotic discs-erythromycin, ciprofloxacin, norfloxacin, streptomycin, and methicillin—were aseptically placed on the inoculated agar surface. The plates were incubated at 37°C for 24 hours. Following incubation, the zones of inhibition around each disc were measured and interpreted to determine the antibiotic resistance profile of the isolates.

Results and Discussion

Five plate screening test: The results of the antimicrobial activity of milk samples evaluated using agar diffusion assays against selected test organisms indicated variable zones of inhibition depending on both the milk source and the bacterial strain tested (Figure-1).

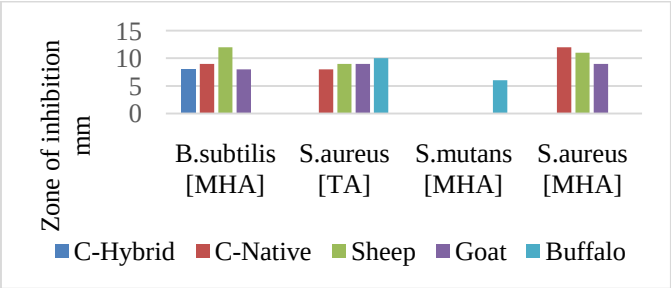


Figure-1: Five Plate Screening Test for milk samples.

The MHA plate inoculated with *Bacillus subtilis* showed inhibition zones of 8mm (hybrid cow milk), 9mm (native cow milk), 12mm (sheep milk), and 8mm (goat milk). The relatively higher inhibition observed in sheep milk suggests a greater presence of antibacterial residues or bioactive compounds compared to other samples. This is consistent with previous findings that milk from different animal sources may contain varying levels of antimicrobial compounds or drug residues due to differences in veterinary treatment practices and metabolism[14].

The test agar (TA) plate inoculated with *Staphylococcus aureus* showed inhibition zones of 9 mm (sheep milk), 8 mm (native cow milk), and 10 mm (buffalo milk), while MHA plates with the same organism showed higher inhibition in native cow milk (12 mm), followed by sheep milk (11 mm) and goat milk (9 mm). These results indicate that *S. aureus* is moderately susceptible to inhibitory substances present in the milk samples, particularly those from native cow and sheep sources. Similar results have been observed in experiments conducted by other researchers[15].

In contrast, *Streptococcus mutans* showed a limited inhibition zone of 6 mm only in buffalo milk, suggesting low antibacterial activity against this organism. Notably, no zones of inhibition were observed in either MHA or TA plates inoculated with *E. coli*. This lack of susceptibility is likely due to the intrinsic resistance of Gram-negative bacteria, attributed to the presence of an outer membrane that restricts the diffusion of many antimicrobial compounds[16]**.**

The results suggest that Gram-positive bacteria (*B. subtilis*, *S. aureus*, and *S. mutans*) were more susceptible to inhibitory substances present in milk samples compared to *E. coli*. Differences in inhibition patterns across milk types may be associated with variation in antibiotic residue levels, bioactive peptides, or natural antimicrobial components such as lactoferrin and immunoglobulins.

Thin-Layer Chromatography (TLC) Analysis**:** The retention factor (Rf) values of milk samples and antibiotic standards were determined following chromatographic separation on TLC plates. TLC is widely recognized as a rapid, simple, and cost-effective screening technique for the detection of antibiotic residues in food matrices[12,17].

Among the milk samples, the Rf value of hybrid cow milk was 0.10, while buffalo milk exhibited an Rf value of 0.20. Sheep milk showed an Rf value of 0.10. The Rf values of the antibiotic standards were recorded as follows: clavulanate (0.06), tetracycline (0.05), ampicillin (0.04), ciprofloxacin (0.08), and doxycycline (0.10) (Table 1). The similarity between the Rf value of sheep and hybrid cow milk (0.10) and that of doxycycline suggests possible co-migration, indicating a potential presence of antibiotic residues in these samples. Such comparative Rf-based screening is commonly used as a preliminary approach for residue detection in animal-derived food products[11,17].

Isolation of bacteria from milk samples: Four bacterial isolates were obtained from different milk samples based on colony morphology and Gram staining. Isolate 1, obtained from native cow milk, was a yellow-pigmented colony identified as Gram-positive cocci arranged in clusters. Isolate 2, also from native cow milk, formed opaque white colonies and was identified as Gram-positive rods. Isolate 3, isolated from goat milk, produced small grey colonies and was identified as Gram-positive rods. Isolate 4, obtained from buffalo milk, formed smooth white colonies and was identified as Gram-positive cocci. These morphological and microscopic characteristics suggest the presence of mixed bacterial populations commonly associated with raw milk, including both coccal and bacillary forms, which may originate from environmental contamination, udder microflora, or handling practices.

Table-1: Rf value of Milk and antibiotic samples.

Milk S ample	Rf	Antibiotics Standard	
Cow (Native)	0.0	Clavulanate	0.05
Cow (Hybrid)	0.1	Tetracycline	0.04
Buffalo	0.2	Amoxycilin	0.08
Sheep	0.1	Ciprofloxacin	0.1
Goat	0.0	Doxycycline	0.06

Determination of Antibiotic Resistance in Bacterial Isolates: Disk Diffusion Technique: The antibiotic susceptibility patterns of the bacterial isolates were evaluated using the disk diffusion method (Table-2, Figure-2). Isolate 1 from native cow milk exhibited sensitivity to ciprofloxacin (38 mm), norfloxacin (31 mm), and streptomycin (22 mm), while showing resistance to methicillin (12 mm). No zone of inhibition was observed for erythromycin, indicating complete resistance. Isolate 2 from native cow milk was sensitive to ciprofloxacin (39 mm), norfloxacin (30 mm), streptomycin (22 mm), and methicillin (18 mm), but resistant to erythromycin (11 mm).Similar results were observed in experiments conducted with Bacillus species[18]. Isolate 3 form goat milk demonstrated sensitivity to all tested antibiotics, including erythromycin (25 mm), ciprofloxacin (30 mm), norfloxacin (26 mm), streptomycin (22 mm), and methicillin (13 mm). Isolate 4 from buffalo milk showed sensitivity to ciprofloxacin (45 mm), norfloxacin (35 mm), streptomycin (23 mm), and methicillin (15 mm), while being resistant to erythromycin (10 mm).

The observed variability in antibiotic susceptibility profiles among isolates may be attributed to differences in environmental exposure, antibiotic usage in dairy animals, and horizontal gene transfer among bacterial populations. Similar findings have been reported in previous studies, where milk-derived bacterial isolates exhibited diverse resistance patterns depending on farm management practices and antimicrobial usage history[16,19].

A consistent observation across all isolates was strong susceptibility to fluoroquinolones, particularly ciprofloxacin and norfloxacin. This suggests that these antibiotics remain effective against most of the isolated strains, likely due to their broad-spectrum activity and lower resistance prevalence in Gram-positive and Gram-variable organisms. In contrast, resistance to erythromycin in multiple isolates may be associated with the widespread occurrence of macrolide resistance genes, which are commonly reported in dairy-associated bacterial flora[20].

Methicillin resistance observed in Isolate 1 further indicates the possible presence of resistant Gram-positive cocci, which may be linked to β-lactam resistance mechanisms such as altered penicillin-binding proteins. Such resistance patterns are of concern in dairy environments, as they may contribute to the dissemination of antimicrobial-resistant bacteria through the food chain[21,22]. These results highlight the presence of both susceptible and resistant bacterial populations in milk samples, emphasizing the importance of continuous monitoring of antimicrobial resistance in the dairy sector[23].

The results demonstrates that the five-plate microbiological screening method, when combined with thin-layer chromatography (TLC), serves as a sensitive and reliable approach for the detection of antibiotic residues in milk samples. The presence of antibiotic residues was confirmed in milk samples obtained from hybrid cow, native cow, and buffalo sources. This indicates the possible exposure of dairy animals to veterinary antibiotics, leading to residue persistence in milk[23,24]. The detection of residues in buffalo milk may also be influenced by dietary factors, including the ingestion of fodder containing natural antimicrobial compounds, although further investigation is required to substantiate this possibility.

Table-2: Antibiotic susceptibility patterns of isolates from milk samples.

Isolates	Antibiotics	Zone of inhibition (mm)	Susceptibility
Isolate-1	Erythromycin	-	
	Ciprofloxacin	38	Sensitive
	Norfloxacin	31	Sensitive
	Streptomycin	22	Sensitive
	Methicillin	12	Resistant
Isolate-2	Erythromycin	11	Resistant
	Ciprofloxacin	39	Sensitive
	Norfloxacin	30	Sensitive
	Streptomycin	22	Sensitive
	Methicillin	18	Sensitive
Isolate-3	Erythromycin	25	Sensitive
	Ciprofloxacin	30	Sensitive
	Norfloxacin	26	Sensitive
	Streptomycin	22	Sensitive
	Methicillin	13	Sensitive
Isolate-4	Erythromycin	10	Resistant
	Ciprofloxacin	45	Sensitive
	Norfloxacin	35	Sensitive
	Streptomycin	23	Sensitive
	Methicillin	15	Sensitive

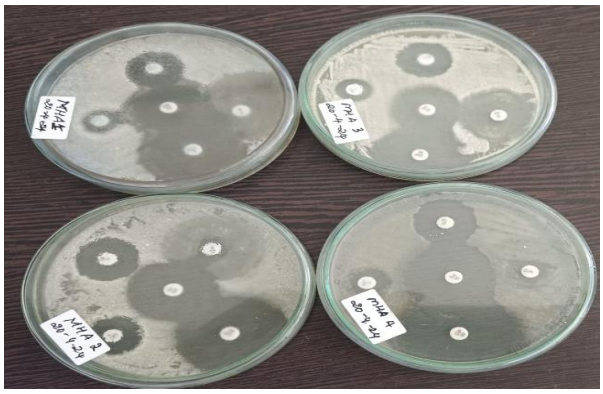


Figure-2: Disk Diffusion results of isolates.

Furthermore, the occurrence of antibiotic residues may be attributed to the failure to adhere to recommended withdrawal periods following antibiotic administration[25]. Withdrawal periods refer to the minimum time required between the last drug administration and the collection of milk intended for human consumption, ensuring that residue levels fall below permissible limits. Non-compliance with these guidelines remains a significant factor contributing to the presence of antibiotic residues in milk.

Conclusion

The findings highlight the increasing prevalence of antibiotic residues in milk, reflecting widespread or improper use of antimicrobial agents in veterinary practices. Additionally, antibiotic susceptibility testing revealed the development of resistance in certain isolates, notably methicillin resistance in an isolate from native cow milk and erythromycin resistance in an isolate from buffalo milk, emphasizing the potential public health risk associated with antimicrobial resistance in dairy-associated bacteria.

To ensure food safety, strict monitoring of milk quality is essential. Proper pasteurization should be implemented, as heat treatment can reduce or inactivate certain antibiotic residues. Furthermore, routine surveillance by food safety and testing laboratories is necessary to ensure that antibiotic residue levels in milk remain below the MRLs established by regulatory authorities.

In conclusion, continuous monitoring and regulated antibiotic use in dairy farming are critical to minimize public health risks associated with antibiotic residues and antimicrobial resistance in the food chain.

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References

1. Economou, V., & Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infection and Drug Resistance, 8*, 49–61.

2. Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences of the United States of America*, 112(18), 5649–5654.

3. Kümmerer, K. (2009). Antibiotics in the aquatic environment-A review-Part I. *Chemosphere*, 75(4), 417–434.

4. Msagati, T. A. M., & Nindi, M. M. (2007). Determination of β-lactam residues in foodstuffs of animal origin using supported liquid membrane extraction and liquid chromatography–mass spectrometry. *Food Chemistry*, 100(2), 836–844.

5. Bártíková, H., Podlipná, R., & Skálová, L. (2016). Veterinary drugs in the environment and their toxicity to plants. *Chemosphere*, 144, 2290–2301.

6. Landers, T. F., Cohen, B., Wittum, T. E., & Larson, E. L. (2012). A review of antibiotic use in food animals: Perspective, policy, and potential. *Public Health Reports*, 127(1), 4–22.

7. Ismail, G., El Hawari, K., Jaber, F., Verdon, E., & Al Iskandarani, M. (2025). Screening methods for antibiotic residue detection in milk: Recent advances, challenges, and regulations. *International Journal of Dairy Technology*, 78(1).

8. Sachi, S., Ferdous, J., Sikder, M. H., & Hussani, S. M. A. K. (2019). Antibiotic residues in milk: Past, present, and future *Journal of Advanced Veterinary and Animal Research*, 6(3), 315–332.

9. Payá, P., Berrada, H., & Mañes, J. (2003). Estimation of the withdrawal period for veterinary drugs used in food-producing animals. *Analytica Chimica Acta*, 483(1–2), 251–257**.**

10. Anika, T. T., Al Noman, Z., Ferdous, M. R. A., Khan, S. H., Mukta, M. A., Islam, M. S., Hossain, M. T., & Rafiq, K. (2019). Time dependent screening of antibiotic residues in milk of antibiotics treated cows. *Journal of Advanced Veterinary and Animal Research*, 6(4), 516–520.

11. Diez, P., Carull, M., Arino, A., Fernandez, J. A., & Centrich, F. (2000). Screening methods for antibiotic residues in food of animal origin. *Journal of AOAC International*, 83(3), 609–615. Google scholar

12. Hancu, G., Simon, B., Kelemen, H., Rusu, A., Mircia, E., & Gyéresi, Á. (2013). Thin layer chromatographic analysis of beta-lactam antibiotics. *Advanced Pharmaceutical Bulletin*, 3(2), 367–371.

13. Kaya, S. E., & Filazi, A. (2010). Application of TLC-bioautography for detection of antibiotic residues in milk. *Food Control*, 21(7), 982–987.

14. Kaya, S. E., & Filazi, A. (2010). Determination of antibiotic residues in milk samples. *Kafkas Üniversitesi Veteriner Fakültesi Dergisi*, 16(Suppl. A), S31–S35. Google scholar

15. Sahoo, S., Behera, M. R., Mishra, B., Sahoo, P., & Kar, S. (2023). Antibiotic-resistant bacteria in bovine milk in India. *Journal of Advanced Veterinary and Animal Research*, 10(1), 21–29.

16. Willey, J. M., Sherwood, L. & Woolverton, C. J. (2013). *Prescott's Microbiology* (9th ed.). McGraw-Hill Higher Education. Google scholar

17. Sherma, J. & Fried, B. (2003). *Handbook of thin-layer chromatography* (3rd ed.). Marcel Dekker. Google scholar

18. Adamski, P., Byczkowska-Rostkowska, Z., Gajewska, J., Zakrzewski, A. J. & Kłębukowska, L. (2023). Prevalence and antibiotic resistance of *Bacillus* sp. isolated from raw milk. *Microorganisms*, *11*(4), 1065.

19. Almashhadany, D. A. (2021). Screening of antibiotic residues in raw milk of cows and buffalos by diffusion assays. *Italian Journal of Food Safety*, 10(3), 9034.

20. CLSI (2023). *Performance standards for antimicrobial susceptibility testing* (33rd ed.; CLSI supplement M100). Clinical and Laboratory Standards Institute.

21. World Health Organization (2017). *Global action plan on antimicrobial resistance*.

22. Mursal, M., Javed, A., Saleem, S., Asia, A., Bukhari, A. K., Kumar, P., Mushtaq, H., & Yousaf, U. (2025). Assessment of antibiotic contamination in milk and its impact on consumer health. *Frontier in Medical & Health Research*, 3(7), 1–10.

23. Fatemi, F., Alizadeh Sani, M., Noori, S. M. A., & Hashemi, M. (2024). Status of antibiotic residues in milk and dairy products of Iran: A systematic review and meta-analysis. *Journal of Environmental Health Science & Engineering*, 22(1), 31–51.

24. Rahman, M. S., Hassan, M. M., & Chowdhury, S. (2021). Determination of antibiotic residues in milk and assessment of human health risk in Bangladesh. *Heliyon*, 7(8), e07739.

25. Hajrulai-Musliu, Z., Uzunov, R., Krluku, M., Jovanov, S., Stojkovski, V., Arapcheska, M., Musliu, D., & Sasanya, J. J. (2023). Determination of multi-class antimicrobial residues and antimicrobial resistance in cow milk and feces samples during withdrawal period. *Animals*, 13(23), 3603.