Antibiotic Residue in Raw Milk Collected from Dairy Farms and Markets in Nepal

S. Pokharel[1!], S. Sedhai[1], R. Thakur[2], M. N. Gautam[1], D. Aryal[1] and N. Upadhyaya[1!]*

[1]Veterinary Standards and Drug Regulatory Laboratory, Kathmandu, Nepal
[2]Agriculture and Forestry University, Chitwan, Nepal
! Equal share as first author

* Corresponding author: drnabinvet@gmail.com

ABSTRACT

Antibiotic residue in food has been a growing public health problem due to the increasing global threats of antimicrobial resistance. Animal products including milk and meats might alos contain residues of antibiotics as administering antibiotics in animals to treat infection is a common practice. However, the level of the presence of residues are not frequently quantified in Nepal. The objective of this study is to estimate the level of residues in milk collected from dairy farms and markets. A total of 935 raw milk samples were collected from 14 districts of 5 provinces namely Koshi, Madhesh, Bagmati, Gandaki and Lumbini province. Samples were tested at the Veterinary Standards and Drug Regulatory Laboratory (VSDRL) from June 2021 to Dec 2022 first using Rapid Test Kit followed by Gentamicin ELISA Kit for quantification. The result showed antimicrobial residue was present in 1.6% of the milk samples (15/935). On further quantification with ELISA, only 4 samples (0.42%) were positive for Gentamicin antibiotic which were below the MRL level. Extensive research with larger sample size and national representative samples are needed to understand the exact scenario of antibiotic residue in milk.

***Keywords**: Antibiotics, Gentamicin, ELISA, MRL*

INTRODUCTION

Milk is a source of high quality protein and bioavailable amino acids, several vitamins, and minerals (Smith et.al., 2021). In low-middle-income countries, milk is an important food source for nurturing the human population with high risks of nutritional deficiencies (Dror & Allen, 2011). Intake of animal food sources including milk can stimulate growth and weight gain in growing children by providing energy, protein, and micronutrients

(Hoppe et al., 2006). In developing countries, milk is the most consumed dairy product and its demand is further increasing with the increased population, urbanization, and awareness among the people (FAO, 2022).

Globally, the use of antibiotics is increasing rapidly for therapeutic and prophylactic purposes along with the growing dairy industry (Kümmerer, 2008). The introduction of antibiotics in 1940s revolutionized medicine by saving millions of lives of peoples from infectious disease. However, soon after the introduction of each new type of antibiotic, bacteria also evolved, gaining the potency to withstand the effect of the antibiotic that previously killed them, and to multiply in the presence of those antibiotics, thus becoming resistant (Pogurschi et al., 2015). Antibiotics are used widely in food-producing animals to improve productivity by lowering the morbidity and mortality (Oliver et al., 2011). The use of antimicrobials in livestock globally is double as compared to humans (Aarestrup, 2012) with approximately 80% of all the food-producing animals receiving medication for part or most of their lives (Lee et al., 2001). Antibiotics are used in the dairy sector not only to control, treat and prevent infection in animals but also for enhancing animal growth and increase feed efficiency (Tollefson & Miller, 2000). Though the use of antibiotics in livestock animals has numerous benefits, its injudicious use in animals leads to the excretion of antibiotics in animal products such as milk, meat, and eggs for a certain period of time as residues (Zhang et al., 2009). These residues have a serious impact on the dairy industry and human health when present above the maximum residue limit (Priyanka et al., 2017). The maximum residue limit is the maximum permissible level of drug or chemical concentration in the food or feeds intended to be used for consumption (Sachi et al., 2019). Various long-term and short-term effects on human health such as allergy, ototoxicity, hepatotoxicity, reproductive disorders, bone marrow toxicity, etc can be seen as a result of the consumption of antibiotic residues (Padol et al., 2015).

The major concern associated with the presence of antibiotic residues above MRL in food products such as milk is the emergence of foodborne AMR. The continuous exposure of bacteria to antibiotic residues can generate antibiotic-resistant bacteria which can complicate the treatment of both human and animal diseases. (Bengtsson-Palme et al., 2018). Antibiotic resistance is a major concern to global health and food security as it is estimated to cause 10 million deaths each year by 2050 (O'Neill, 2014).

In Nepal, the dairy industry contributes to 9% of the total GDP (two third of Livestock GDP). Dairy industry in Nepal has also grown rapidly in the past years with a total production of 2.47 million metric tons of milk in 2020/21 which shows an increment of 7.77% compared to the last fiscal year (DLS, 2020). Several studies on the antibiotic residue in meat and milk of different districts has been done before by Thapaliya et al., 2013, Khanal et al., 2018, Gompo et al., 2020. There is a government regulatory laboratory; Veterinary Standards and Drug Regulatory Laboratory (VSDRL), with a mandate of

testing drug residue in meat, milk and egg. Also, Nepal has signed the signatory of Jaipur Declaration on AMR (2011), is a member of stakeholders of tripartite association (WHO, WOAH and FAO) for of Global Action Plan for control of AMR and has defined focal point for antimicrobial use, it is important for the assessment of antibiotic residues in food and the environment to know about the status of antimicrobial use and to control the emerging risk of antimicrobial resistance. Thus, this study aims to provide a recent data on the antibiotic residue in milk of major dairy hub of Nepal in order to have a picture of antibiotic residue scenario in animal products in the country.

MATERIALS AND METHODS

The present study was conducted at the Veterinary Standards and Drug Regulatory Laboratory (VSDRL) from June 2021 to Dec 2022 as a part of routine testing of the laboratory.

Sample collection

A total of 935 raw milk samples were collected from 5 provinces of Nepal using random sampling method. Out of the total samples, 36 samples were collected from Koshi province, 99 samples were collected from Madhesh province, 460 samples were collected from Bagmati province, 247 samples were collected from Gandaki province and 93 samples were collected from Lumbini province. Samples were collected in sterile collection tubes and transported to the laboratory in icebox where they were further stored at -20^{0}C before testing.

Sample processing

Samples were processed within seven days of sample collection. The raw milk samples were heated in a water bath at 82^{0}C for 2 minutes to destroy heat labile natural inhibitors and microorganisms contaminated in raw milk as per the guidelines provided in test kit.

Test for presence and absence of drug residue

The milk samples were tested using Rapid Test Kit for determination of drug residues in milk and milk products by Asian Medic Co. Ltd., Thailand. The test kit has 91.7% accuracy, 100% sensitivity and 90.5% specivity in detecting the presence of 12 kinds of antibiotic drug residues in milk.

Procedure: 0.1 ml of milk sample was added to the prepared tubes and 0.1 ml of UHT fresh milk was added into another prepared tube for negative control. All the tubes were incubated for 2 hours 30 minutes in water bath at 64^{0}C, keeping medium in the tube under water level. Then, the colour change of medium in sample tubes were observed. A clear colour change from purple to yellow indicated the absence of antimicrobial residues. A purple colour indicated the presence of antibiotic residues and the level of purple colour

indicated the quantity of drug residues.

ELISA for the quantification of gentamycin in positive samples

The positive samples from rapid test were further tested with Gentamicin ELISA kit (Elabscience Biotechnology Inc., United States) for quantification of Gentamycin antibiotics in the milk samples.

Preparation of sample for ELISA: 1 gm of the sample was combined with 1 ml of 1% trichloroacetic acid solution and mixed by vortex for one minute. Then the sample was centrifuged at 4000 rpm for 5 minutes at room temperature. 950µl of sample solution was added to50 µl of the obtained intermediate liquid and was mixed properly. The final mixture was then used for analysis.

Assay procedure: Before the assay, all the reagents and samples were brought to room temperature and a record of sample wells and standard wells was prepared. 50 µl of standard solution and sample solution were placed in respective wells. 50 µl of HRP conjugate and 50 µl of antibody working solution were added to each well and were mixed thoroughly. The plate was then covered and incubated for 30 minutes at 25^0C. After 30 minutes, the cover was removed and the wells were washed with 260 µl of wash buffer for 4 times. After this, the plate was dried by inverting and patting it against thick clean absorbent paper until the wells were dried and no bubbles were seen. Then 50 µl of substrate reagent was added to each well and the plate was oscillated for 15 seconds to ensure proper mixing. The plate was then incubated for 15 minutes at 25^0C in the dark. Finally, 50 µl of stop solution was added to each well and the plate was gently oscillated for 10-15 seconds to ensure the complete mixing of the reagents.

The optical density was measured at 450nm using a microplate reader (Multiscan ex, Thermo, USA). The final results were calculated by obtaining the optical density values and calculating the absorbance percentage.

$$Absorbance\ \% = \frac{average\ absorbance\ of\ standard\ or\ sample}{average\ absorbance\ of\ 0\ ppb\ standard\ solution} x100\%$$

A calibration curve was plotted between the standard concentration and optical density. The obtained values were cross verified using a software (Ridasoft Win.NET)

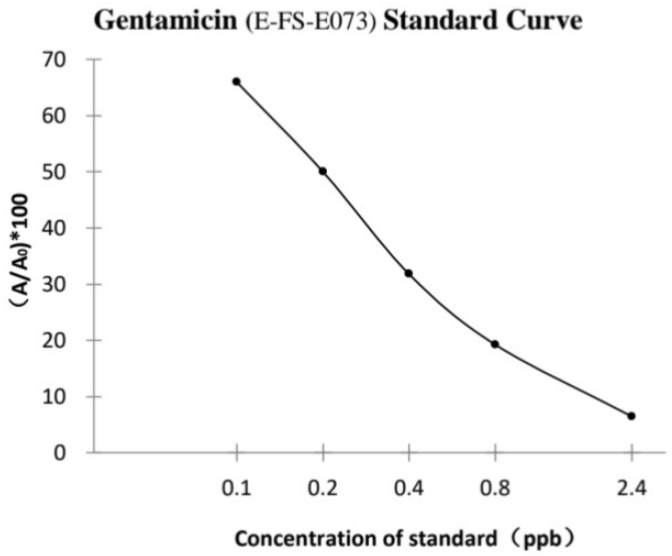


Figure 1: Gentamycin standard curve

Data analysis

The data were entered in Microsoft Excel version 2303 and data were visualized in bar graphs and tables using MS Excel and the maps were created using QGIS version 3.22.5.

RESULTS AND DISCUSSION

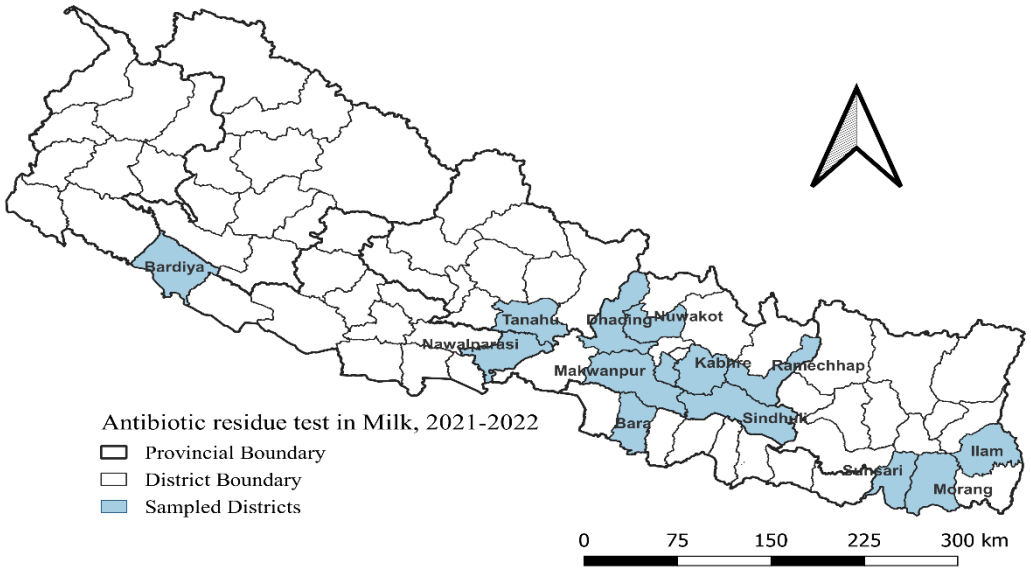
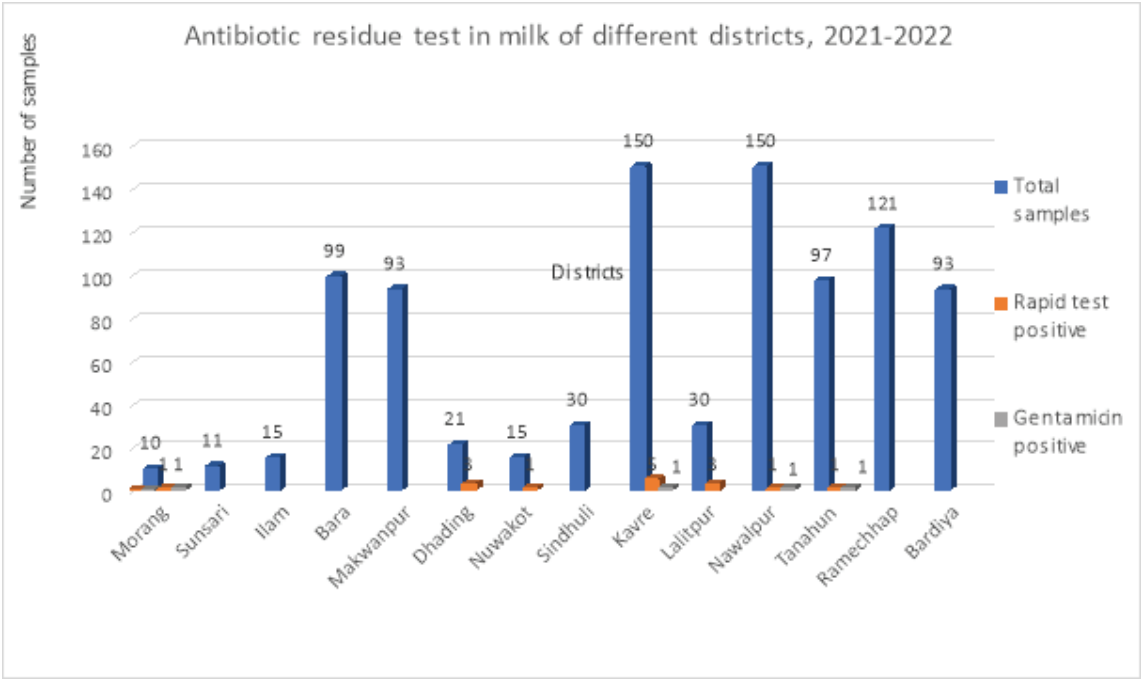


Table 1: Milk samples collected from different provinces

Province	Number of samples collected	Number of samples positive (Rapid test)	Percentage of sample positive
Bagmati	460	12	2.61
Gandaki	247	2	0.81
Koshi	36	1	2.78
Lumbini	93	0	0.00
Madhesh	99	0	0.00
Total	935	15	1.60

A total of 935 milk samples were tested for the presence of antibiotic residue. Overall, 1.6 % (n=15) of samples were positive for the presence of antibiotic residue. Milk samples from Bagmati province were seen highly positive.



Out of 14 districts sampled, Kavre district had highest number of samples (n=5) positive for antibiotics in milk, followed by Dhading and Lalitpur (n=3 each).

The positive samples were further tested with Gentamicin ELISA test kit. Out of 15 positive samples, four samples were positive for Gentamicin antibiotic. The Gentamicin positive samples were from Kavre, Morang, Nawalpur and Tanahu. The antibiotic level ranged from 2.25 to 3 ppb which is less than recommended Maximum Residue Limit (MRL) of 100 ppb for Gentamicin in milk as per the European Union and Codex Alimentarius Commission.

This study was done on laboratory data of recent two years of VSDRL. The rapid test result shows an overall of 1.6% sample positive for presence of antibiotics in milk. The result is less than other similar studies. Thapaliya et al., 2013 found 17.3% of market milk positive for antibiotic residues that were collected from 4 districts of Nepal. Similarly, Khanal et al., 2018 found 23% milk samples, positive for the presence of antibiotic residues while Bhusal et al., 2020 found a 6% prevalence of antibiotic residues in milk samples from Kathmandu valley. Gompo et al., 2020 detected antibiotic residues in 55% of the milk samples collected from the Kaski district. The higher percentage positive samples in milk of Kaski and Kathmandu districts may be because of intensive farming practices in such urban areas where the use of antibiotics might be relatively higher. Likewise, our study also shows a higher percentage of sample positive in Dhading (14%), Lalitpur (10%) and Morang (10%) districts. The decrease in overall percentage of antibiotic positive milk samples also may be the result of awareness among the farmers regarding the withdrawal period of drugs, prudent use of antimicrobials by veterinarians and number of samples. However, the data is likely to be underestimated as the major milk producing districts are not included in the study. Also, two provinces are not sampled at all and only one district representing the Lumbini province is included in the study. This demands the need for an extensive study covering all regions of the country to have data and to know about the actual scenario of antibiotic residue in milk.

CONCLUSION

Antimicrobial residue in milk is one of the growing issue for the human health. This study provides a recent data to know the current situation of antimicrobial residue, and provide a baseline for the further study on antimicrobial use in animals. The result shows the presence of antimicrobial residue in milk in 1.6% of the 935 samples collected from different 14 districts of Nepal. On further quantification with ELISA, only 4 samples were positive for Gentamicin antibiotic which were below the MRL level. This comparatively less percent positive milk for antibiotic residue than previous studies may be the result of the proper use of antimicrobials by veterinarians, increased awareness among the farmers regarding the withdrawal period of drugs, and increased testing of drug residues in milk. However, extensive research with larger sample size including highly productive districts and representing all the provinces is needed to know about the exact scenario of antibiotic residue in milk.

REFERENCES

- Aarestrup, F. (2012). Sustainable farming: Get pigs off antibiotics. Nature, 486(7404), 465–466. https://doi.org/10.1038/486465A
- Bengtsson-Palme, J., Kristiansson, E., & Larsson, D. J. (2018). Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiology Reviews, 42(1). https://academic.oup.com/femsre/article-abstract/42/1/fux053/4563583

- Bhusal, D. R., Chhetri, B., & Subedi, J. R. (2020). Determination of Antibiotics Residues in Milk Samples Collected in the Different Sites of Kathmandu, Nepal. Asian Journal of Dairy and Food Research, OF. https://doi.org/10.18805/AJDFR.DR-186
- Britannica. (2022). Milk. Encyclopedia Britannica. https://www.britannica.com/topic/milk
- Dror, D. K., & Allen, L. H. (2011). The Importance of Milk and other Animal-Source Foods for Children in Low-Income Countries. Http://Dx.Doi.Org/10.1177/156482651103200307, 32(3), 227–243. https://doi.org/10.1177/156482651103200307
- FAO. (2022). Gateway to dairy production and products. https://www.fao.org/dairy-production-products/products/en/
- Gompo, T. R., Sapkota, R., Subedi, M., Koirala, P., & Bhatta, D. D. (2020). Monitoring of Antibiotic Residues in Chicken Meat, Cow and Buffalo Milk Samples in Nepal. International Journal of Applied Sciences and Biotechnology, 8(3), 355–362. https://doi.org/10.3126/IJASBT.V8I3.31314
- Hoppe, C., Mølgaard, C., & Michaelsen, K. F. (2006). Cow's Milk and Linear Growth in Industrialized and Developing Countries. Annual Review of Nutrition, 26, 131–173. https://doi.org/10.1146/ANNUREV.NUTR.26.010506.103757
- Khanal, B. K. S., Sadiq, M. B., Singh, M., & Anal, A. K. (2018). Screening of antibiotic residues in fresh milk of kathmandu valley, nepal. Journal of Environmental Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes, 53(1), 57–86. https://doi.org/10.1080/03601234.2017.1375832
- Kümmerer, K. (2008). Pharmaceuticals in the environment: sources, fate, effects and risks. Springer Science and Business Media. https://books.google.com/ks?hl=en&lr=&id=hS1EAAAAQBAJ&oi=fnd&pg=PR28&dq=+K%C3%BCmmerer+HTK.+Pharmaceuticals+in+the+environment+%E2%80%93+sources, + fate, +effects + and + risks. +Aquat+Toxicol.+ 2001%3B71:391%E2%80%932.+% 5B Google + Scholar %5D& ots = wNEHwHlCgz&sig = GNJXMKUeDEDncctmyKc7R3aq2c4
- Lee, M. H., Lee, H. J., & Ryu, P. D. (2001). Public Health Risks: Chemical and Antibiotic Residues. Asian-Australasian Journal of Animal Sciences, 14(3), 402–413. https://doi.org/10.5713/AJAS.2001.402
- MoALD. (2022). Statistical Information on Nepalese Agriculture. https://moald.gov.np/wp-content/uploads/2022/07/STATISTICAL-INFORMATION-ON-NEPALESE-AGRICULTURE-2077-78.pdf
- Oliver, S. P., Murinda, S. E., & Jayarao, B. M. (2011). Impact of antibiotic use in adult dairy cows on antimicrobial resistance of veterinary and human pathogens: a comprehensive review. Foodborne Pathogens and Disease, 8(3), 337–355. https://doi.org/10.1089/FPD.2010.0730
- O'Neill, J. (2014). Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. The Review on Antimicrobial Resistance.
- Padol, A. R., Malapure, C., Domple, V. D., & Kamdi, B. (2015). Occurrence , Public Health Implications and Detection of Antibacterial Drug Residues in Cow Milk. Environ. We Int. J. Sci. Tech, 10, 7–28.
- Priyanka, P., Sheoran, M., & Ganguly, S. (2017). Antibiotic residues in milk-a serious public health hazard. J Environ Life Sci, 2(4), 99–102. https: //www.academia.edu/download/55174563/Priyanka_et_al.2017_JELS_Online.pdf
- Sachi, S., Ferdous, J., Sikder, M. H., & Azizul Karim Hussani, S. M. (2019). Antibiotic residues in milk: Past, present, and future. Journal of Advanced Veterinary and Animal Research, 6(3), 315. https://doi.org/10.5455/JAVAR.2019.F350
- Smith, N. W., Fletcher, A. J., Hill, J. P., & McNabb, W. C. (2021, December 20). Modeling the Contribution of Milk to Global Nutrition. Frontiers. https: //doi.org/10.3389/fnut.2021.716100
- Thapaliya, M., Karki, T. B., & Sedai, D. (2013). Sulfonamides and Penicillin Residue in Market Milk. Journal of Food Science and Technology Nepal, 8, 60–64. https://doi.org/10.3126/JFSTN.V8I0.11751
- Tollefson, L., & Miller, M. A. (2000). Antibiotic use in food animals: controlling the human health

impact. Journal of AOAC International, 83(2), 245–254. https://academic.oup.com/jaoac/article-abstract/83/2/245/5656315

- Virto, M., Santamarina-García, G., Amores, G., & Hernández, I. (2022). Antibiotics in Dairy Production: Where Is the Problem? https://doi.org/10.3390/dairy3030039
- Zhang, H., Ren, Y., & Bao, X. (2009). Simultaneous determination of (fluoro) quinolones antibacterials residues in bovine milk using ultra performance liquid chromatography–tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis, 49(2), 367–374. https://www.sciencedirect.com/science/article/pii/S0731708508005803