



Research Article

Monitoring of Antibiotic Residues in Chicken Meat, Cow and Buffalo Milk Samples in Nepal

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Keywords: Antibiotic residue; chicken meat; milk samples; maximum residue limit (MRL); Nepal

Abstract

In animal products, antibiotic residue is a serious concern for public health in the Himalayan nation, Nepal. A cross-sectional study on antibiotic residues in chicken meat, milk samples of cows, and buffaloes was conducted in selected districts of different provinces of Nepal. Altogether 126 chicken meat samples, 42 each from Kailali (Farwestern Province), Kaski (Gandaki Province), and Nuwakot (Bagmati Province) districts were tested for tetracycline, sulphonamides, penicillin, gentamicin, streptomycin residues by the ELISA method. At Kaski district approximately 57 % (24/42), 50 % (21/42) and 83% (35/42) of meat samples were tested positive for gentamicin (mean-ppb: 11.5 µg/Kg/L), tetracycline (mean-ppb: 1.44 µg/Kg/L), and fluoroquinolones (mean-ppb: 11.46 µg/ Kg/L) residues respectively. In Kailali and Nuwakot district, 48% (20/42) and 50 % (21/ 42) of meat samples deemed positive for sulphonamides (mean-ppb: 15.9 µg/ Kg/L) and penicillin (mean-ppb: 0.39 µg/ Kg/L) residues, respectively. Of the total 168 tests performed in milk samples from Kavre district, 55% (93/168) of the milk samples were tested positive to antibiotic residues for gentamicin, streptomycin, and sulphonamides. The chicken meat samples had antibiotic residues within the national maximum residue limit (MRL); however, milk samples exceeded the national MRL for sulphonamides residues (mean-ppb: 26.44 µg/ Kg/L). The effective surveillance for antibiotic residues in animal products should be implemented strictly in Nepal.

Introduction

With the increase in the demand for protein consumption for humans, the rapid development of animal agriculture took place in the world (Lee *et al.*, 2018). Similarly, the use of antibiotics has also been increased for the improvement of animal health and welfare (Virolainen *et al.*, 2008). However, the overuse of antibiotics in the food animals not only increases the cost of production, but also creates public health concern. For instance, it causes in some case allergic reactions, imbalance of intestinal microbiota, and the potentiality of developing antibiotic-resistant bacteria (Virolainen *et al.*, 2008; Van Boeckel *et al.*, 2015).

In Nepal, about 12.4 percent of the national GDP is contributed by the livestock and aquaculture; poultry singly contributes around 4 percent of national GDP (Government of Nepal & Ministry of Livestock Development, 2017). Nepal has more than 25 million ruminants (cattle, buffaloes, goat, sheep) and 1.4 million pigs and more than 72 million poultry birds. The total per capita consumption of meat, milk, and eggs is 11kg, 73 liters, and 44 eggs, respectively (CBS, 2015; Ghimire, 2017; AITC, 2019). The per-capita meat consumption of poultry meat alone contributes to is 4.1 kg. The uses of antibiotics in poultry

and livestock kept rising during the last decade, and still, the counter sale of poultry medicine is available in Nepal. There is minimal information, however, on the use of antimicrobials in animals, including poultry sectors. The total amount of antimicrobials consumption in animal sectors in 2018 was about 91.1 tons in Nepal (VSDRL Technical Bulletin, 2020). In 2019 alone, around 48 tons of antibiotics were consumed in animal sectors that includes third and fourth generation cephalosporin (9.1 tons), aminoglycosides (2.82 tons), amphenicols (0.14 tons), fluoroquinolones (6.53 tons), macrolides (1.8 tons), penicillin's (2.2 tons), sulphonamides (1.3 tons), tetracycline's (9.7tons), nitrofurans (1.9 tons) and others 2.2 ton (VSDRL Technical Bulletin, 2020).

Antibiotics, when administered, converted to metabolites and excreted from the system, but some portions remain in the meat, milk, and eggs known as antibiotic residue (Nisha, 2008; Sachi *et al.*, 2019). There are some studies related to antibiotic residue in Nepal with limited samples and with the various techniques. The method used for these studies were based on rapid diagnostic tests, microbiological tests, immunological tests, and instrumental analytical methods. Prajapati *et al.* (2018) found that 62% (57/92) broiler meat samples were positive to antibiotic residue in by ELISA methods that were collected from Chitwan, Kathmandu, and Kaski. In another study conducted at Kavre and Kailali district of Nepal, the prevalence of antibiotic residue (Penicillin/Tetracycline/Aminoglycosides/Macrolides/Sulfonamides) in marketed meat was found to be 22% (12/55) by the application rapid test methods (Raut *et al.*, 2017). Similarly, a separate study conducted at Gorkha, Parsa, Chitwan, and Kathmandu districts with broiler meat detected as high as 40% (32/80) penicillin, aminoglycoside, and Tetracyclines by the rapid test kit (Rawal, 2015). Another cross-sectional study conducted in Kathmandu valley in 2019, by the use of disc assay methods, detected 13.33 (8/60) poultry meat samples by Sapkota *et al.* (2019).

In a study in fresh milk sample of Kathmandu Valley, high-performance liquid chromatography (HPLC) analyses detected 81% samples positive for amoxicillin (68–802 mg/kg), 41% for sulfadimethoxine (31–69 mg/kg), 27% for penicillin G (13–353 mg/kg), and 12% for ampicillin (0.5–92 mg/kg) (Khanal *et al.*, 2018). Another study in 2013, the prevalence of antibiotic residue (penicillin/sulfonamide) in milk samples from Kathmandu valley and Kavre was found to be 17.3% by the application of rapid diagnostic test kit provided by Ministry of Public Health Thailand (Thapaliya *et al.*, 2014).

For the detection and quantification of the antibiotic residue in food products, several methods like the microbiological

test, immunological test (ELISA), HPLC, thin layer chromatography (TLC), are available, but they have their limitation (Menkem *et al.*, 2019). The rapid diagnostic test kit methods are fast and comparatively easy, but they have validity issues. The microbial tests are rapid and quickly conducted but they have reduced sensitivity and specificity (Chen *et al.*, 2017). The instrumental analytical methods such as HPLC are highly precise, but they need sophisticated and expensive machines with tedious sample preparations (Chen *et al.*, 2017). Out of the various methods, Enzyme Link Immunoassay Assay (ELISA) provides the excellent sensitivity and selectivity for the finding the antibiotic residue in meat sample and its pretty easiness in application (Ramatla, 2017).

Since most of the previous studies were focused on a specific location of the country with a limited sample size, we decided to select districts from the different provinces of Nepal with a representative sampling. Our main objective was to determine the amount of various antibiotic residues present in chicken meat and milk samples sold for human consumption.

Material and Methods

Study Design and Site Selection

A cross-sectional study was conducted from January 2019 to January 2020 in selected districts of Nepal. The districts selected were Kailali (Far-western Province), Kaski (Gandaki Province), Nuwakot (Bagmati Province), and Kavre (Bagmati Province). The site selection was based on the population of poultry (Kaski, Kailali, and Nuwakot) and the location of dairy pockets (Kavre) in the nation. The study areas are shown in Fig. 1.

Sample Size and Sample Collection

We collected a total of 126 (42X3) chicken meat samples: 42 samples each from Kailali (Dhangadi), Kaski (Pokhara), and Nuwakot (Bidur) districts of Nepal during January 2019 to June 2019. The meat samples were collected from the local meat retailers, consisted of different organs of poultry such as the heart, liver, and muscle.

Next, a total of 84 (42X2) milk samples: 42 each from Kavre (Banepa) and Kavre (Panuti) district of Nepal, were collected during August 2019 to November 2019. The sample consisted of milk of cows and buffaloes collected from community dairies located at Kavre district.

The number of total samples collected was purposive; however, the method of sampling was based on simple random sampling selecting the areas with the highest number of meat shops for meat sampling and selecting the dairy pockets in the districts for the milk sampling.

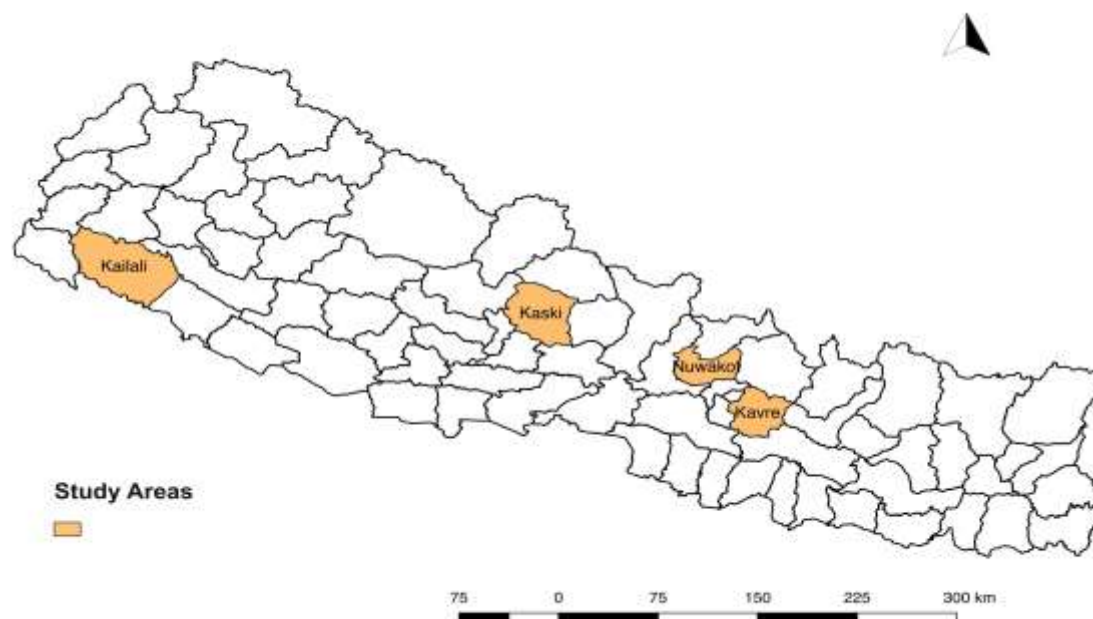


Fig. 1: A map of Nepal with study district highlighted.

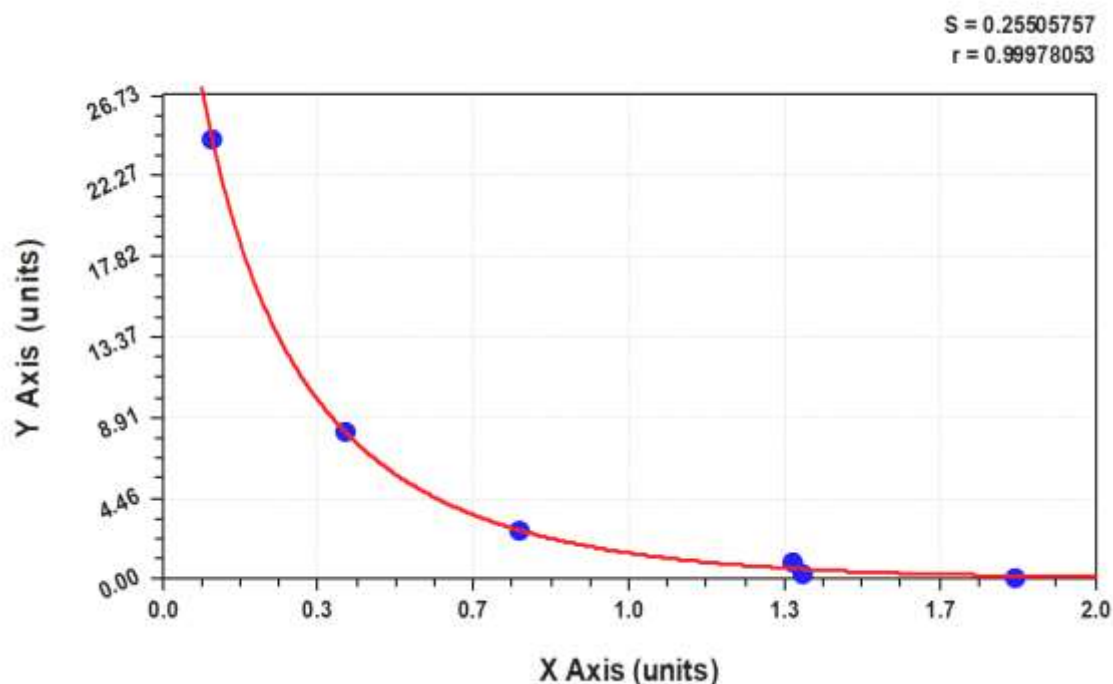


Fig. 2: A curve after fitting the logistic model to determine the antibiotic concentrations for the given OD value.

Sample Preparation and Sample Testing

About 20 gm of meat samples were collected and dispatched to Central Veterinary Laboratory (CVL), Kathmandu with appropriate cold chain maintenance. 2 gm of the meat sample was adequately minced by mortar & pestle to make a working sample. Then the sample was mixed with the 3ml of distilled water and 6 ml extraction solution purchased from CUSABIO, China. The mixture

was centrifuged to 4000 r/min for 5 min at room temperature. 3ml of the supernatant was transferred to new tubes and dried. Then 1 ml of N-hexane and 1 ml of sample diluent added on and was shaken vigorously for 30 seconds. The solution was then centrifuged at 4000 r/min for 5 min. 100 microliter of the aliquot was collected without any solid particles in it. Then the aliquot was subjected to the ELISA process according to the manufacturer's instructions. The necessary process was almost the same for all the antibiotics

panels (tetracycline's, penicillin's, gentamicin, fluoroquinolones, and sulphonamides) for meat testing.

For the milk sample preparation, 100 µL of the sample was added to 900 µL of sample diluent and mixed properly. Then 50 µL of the mixture was taken for future analysis by ELISA. The process was strictly carried out based on the test protocols provided by CUSABIO, USA, 2019.

Each control and samples were duplicated for every batch of testing to increase the precision.

Every collected meat sample was divided into five parts and tested for five different antibiotics: gentamicin, sulphonamides, fluoroquinolones, penicillin, and fluoroquinolones. The detailed description of the test results, as depicted in Table 1. Likewise, the milk samples were divided into four replicates (42 X 4 = 168) and tested for gentamicin, streptomycin, and sulphonamides residues (Table 2).

Statistical Analysis

The ELISA plates were subjected to ELISA readers (Thermo Scientific™, Multiskan™ FC, microplate photometer) to record each sample's OD values at Central Veterinary Laboratory, Kathmandu. The average OD values of each sample recorded were analyzed, and the corresponding concentration antibiotic residue was calculated by the software (Curve Expert 1.4) provided by cusabio.com (Fig 2). The model used to calculate the corresponding concentrations of antibiotic residues were according to the equation 1.

Model1: Logistic Model: $y = a / (1 + b * \exp(-cx))$,
 (Eq. 1),

where, $a = -26.335413$, $b = -1.5588548$, $c = -2.4912812$ are the default coefficients/constants, 'x' is the average ODs value recorded and 'y' is corresponding antibiotic residue concentrations (ppb). After multiple iterations, the best model was selected (Model1) with the highest correlation value ($r = 0.99$ and $s = 0.26$), while adjusting the standards (controls) concentrations provided within the kit. The fitting of the curve of the model is shown in figure no 2. Next, the variables such as the location of samples collected, types of samples were analyzed, and data were visualized by the use of R Studio Version 1.2.5.2019.

Results

Altogether 210 tests were conducted to screen the presence of antibiotic residues in meat samples. In Kaski district, of the 42 tests ran each for gentamicin, tetracycline, and fluoroquinolones, 24 (57.14%), 21 (50.00%), and 35 (83.33%) were tested positive respectively. Similarly, in the Kailali district, of the 42 tests performed for sulphonamides, 20 (47.62%) of the tests were deemed positive. Likewise, in Nuwakot district, of the 42 tests conducted for penicillin, 21 (50.00%) of the tests were positive. The detailed description of each organ tested with the mean antibiotic residue concentrations in parts per billion(ppb) or µg/ Kg/L for each organ is described in Table 1. None of the antibiotic residues exceed the national Maximum Residue Limit (MRL) of 50 ppb or 50 µg/ Kg/L (VSDRL, 2015).

The overall antibiotic concentrations vary within different parts (heart, liver, muscle) of chicken carcass which are visualized by the box-plots (Fig.3).

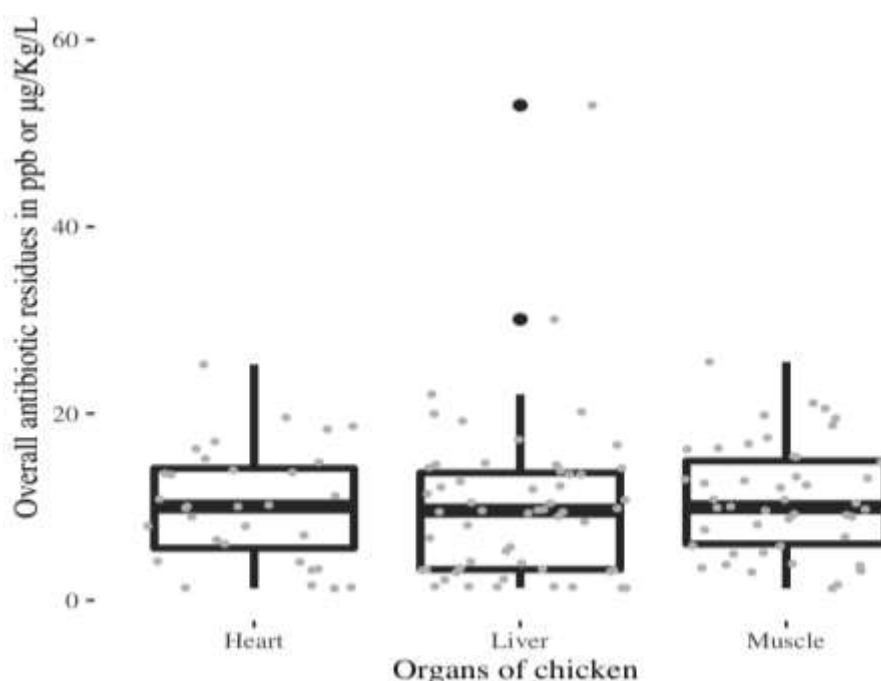


Fig. 3: The distribution of the overall antibiotic concentrations in different organs of chicken.

Table 1: Antibiotic residue tests result for the meat sample collected from different districts of Nepal

Samples collected (District)	Antibiotics	Total sample tested	Total Positive	Positive			Mean ppb (µg/ Kg/L)			Exceed National MRL limit
				Heart	Liver	Muscle	Heart	Liver	Muscle	
Kaski (Pokhara)	Gentamicin	42	24 (57.14%)	5 (11.9%)	11 (26.19%)	8 (19.01%)	11.5	10.88	11.01	No
Kaski (Pokhara)	Tetracycline	42	21 (50.00%)	5 (11.9%)	10 (23.81%)	6 (14.23%)	1.16	1.44	1.48	No
Kaski (Pokhara)	Fluoroquinolone	42	35 (83.33%)	8 (19.04%)	14 (33.33%)	13 (30.95%)	10.87	12.54	10.99	No
Kailali (Dhangadi)	Sulphonamides	42	20 (47.62%)	6 (14.3%)	3 (7.14%)	11 (26.19%)	15.97	15.87	16.12	No
Nuwakot (Bidur)	Penicillin	42	21 (50.00%)	4 (9.52%)	6 (14.29%)	11 (26.19%)	0.14	0.13	0.12	No
Grand Total		210	121 (57.62%)	31 (14.76%)	44 (20.95%)	49 (23.33%)				

*Figures in the parentheses indicate percentage

Table 2: Antibiotic residue tests result for the milk sample collected from different districts of Nepal

Samples collected (District)	Antibiotic	Species	Total Sample tested	Positive (%)	Mean ppb (µg/ Kg/L)	Exceed National MRL
Kavre (Panuati)	Gentamicin	Cow milk	25	11 (44.00%)	4.29	No
Kavre (Panuati)	Gentamicin	Buffalo milk	17	10 (58.82%)	4.43	No
Kavre (Dhulikhe)	Streptomycin	Cow milk	47	23 (48.94%)	28.94	No
Kavre (Dhulikhe)	Streptomycin	Buffalo milk	37	28 (75.68%)	43.32	No
Kavre (Panuti)	Sulphonamides	Cow milk	25	10 (40.00%)	26.2	Yes
Kavre (Panuti)	Sulphonamides	Buffalo milk	17	11 (64.71%)	26.68	Yes
Grand Total			168	93 (55.36%)		

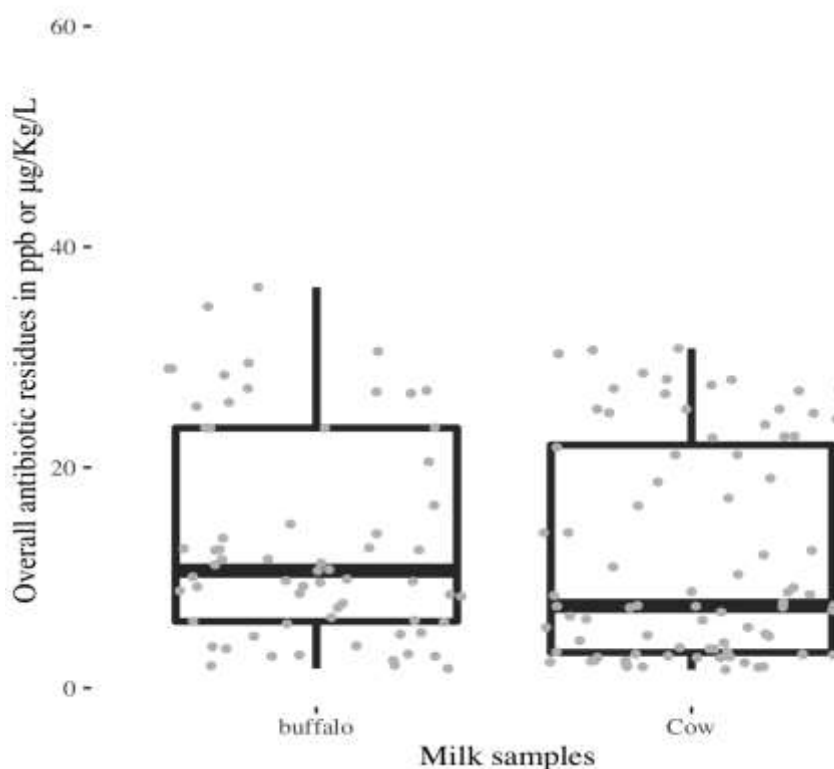


Fig. 4: The distributions of antibiotics concentrations in cow and buffalo milk samples.

Of the total 168 tests performed in the milk samples, of Kavre district, 44.00% (11/25) cow milk samples and 58.82% (10/17) buffalo milk samples were tested positive to gentamicin, 48.94% (23/47) cow milk and 75.68% (28/37) buffaloes milk samples were positive to streptomycin, and 40.00% (10/25) of cow milk samples and 64.71% (11/17) of the buffalo milk samples were tested positive for the sulphonamides (Table 2). The mean ppb ($\mu\text{g}/\text{Kg}/\text{L}$) of sulphonamides residue in the cow milk and the buffalo milk was 26.9, and 26.68 ppb respectively exceed the national MRL (VSDRL, 2015) that is shown as in Table 2.

The overall antibiotics concentrations between the cow and buffalo milk samples also varied widely. The comparison is depicted by the boxplots in the Fig. 4.

Discussion

The antibiotic residue ranges from 50 to 83 percent of the tested in meat samples and 44 to 75 percent in milk samples. The mean ppb of antibiotics residue varies with the locations and type of samples tested (Hind, 2014; Ghasemi *et al.*, 2014). For instance, the percentage positivity of fluoroquinolones was as high as 83.33% (35/44) in the Kaski districts. This is similar to a study conducted by Prajapati *et al.* (2018), who detected a higher percentage of ciprofloxacin and enrofloxacin in poultry meat. On the other hand, the poultry industry in Kaski is rising for a decade, and it is ranked fifth in the nation after Kathmandu in the broiler population (CBS, 2015). Among all the antibiotics in meat, the penicillin residue is of lowest mean concentrations because this group of drugs is less frequently

used in the broiler meat nowadays (personal communication with poultry clinicians of Nepal).

The antibiotic residue ranges from the mean ppb of 1 to 16 in different districts, but none of them exceeds the national MRL limit. There is a concern among the public health experts that repeated consumption of antibiotic-rich chicken could lead to alteration of gut microflora that potentially leads to the development of resistant bacterial strains in humans (Nisha, 2008). Generally, broiler chickens are marketed around 40 to 50 days in Nepal. At this stage, many of them are under the antibiotics medication. The farmers are forced to treat and sell the birds at this stage because they are susceptible to many diseases and bacterial and viral infections (Gompo *et al.*, 2019).

The concentrations of different antibiotic residues vary within the heart, liver, and muscle of chicken tissue though the overall mean concentration remained the same. The liver has a higher range of antibiotics and has few outliers than muscle and heart. The finding is in line with the study conducted by Shahid *et al.* 2007; Hind, 2014).

The presence of antibiotic residues in milk of both the cow and buffaloes is a public health concern, though this study does not represent the total population status of milking cows and buffaloes in Nepal. Similar to the survey by Khanal *et al.* (2018) found the use of penicillin groups is as high as 45% and 32% of the sulpha groups of drugs in milk samples collected in Kathmandu valley by the use of HPLC methods. All these groups of antibiotics, such as gentamicin, streptomycin, penicillin, and sulphonamide, are

used to treat clinical mastitis in milking animals. Moreover, mastitis is a significant concern for the economic loss in the milking animals of Nepal. The farmer's knowledge of the withdrawal period of drugs is limited, and those who knew it were reluctant to hold the milk during the treatment because the family doesn't like to lose money, not by selling it (Ng *et al.*, 2010). It may be the reason for the detection of antibiotic residue in the milk samples of cows and buffaloes in Nepal.

Conclusion

The detection of antibiotic residues in chicken meat and milk samples indicates either overuse of the antibiotics or the practice of selling the animal and their products without surpassing the withdrawal period. Though the quantity of antibiotics detected were below the national maximum residue limit (NMRL), the repeated exposure could be the public health hazard. We suggest a further detailed study in this area.

Recommendations

The only standalone document for the regulation of antibiotics at present in Nepal is the "The Drug Act, 1978" which does not mention anything related to control of veterinary medicine. So, the drug act should be revised to address the current gaps in regulating the use of medicines in the animal industry. There is no approved national action plan for antimicrobial use in animal health. The national microbial standard for meat, milk, egg, and MRL of the veterinary drugs is the current living document; however, its implementation part still is lacking. For this, a comprehensive plan for the surveillance of antibiotic use, awareness program to the concerned parties and the general public should be made available.

Authors' contribution

Tulsi Ram Gombo (TRG) conceived the idea, went to field to collect samples, lead the lab testing, analyzed the data, wrote and revised the manuscript. Ramchandra Sapkota (RC) went to field to collect samples and performed lab testing. Manita Subedi (MS) helped in lab testing. Pragya Koirala (PK) and Diker Dev Bhatta (DDB) provided resources. All authors agreed the papers for the final submission.

Conflict of Interest

The authors declare that there are no conflicts of interest with this research.

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