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Research Article

Rapid Detection of Tetracycline Residues in Chicken

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Abstract

Antibiotic residues in meat are a serious public health concern as a result of its harmful effects on consumer health. This study aimed at estimating the residues levels of commonly used antibiotics (Tetracycline) in chicken samples using two analytical methods; Rapid Screening Kit and HPLC.

Twenty chicken samples were collected from various meat shops of Kathmandu valley. Qualitative and semi-quantitative analyses with Quicking Tetracycline rapid Test kit, which detect tetracycline above 100 ppb in tissue revealed that two samples were positive for antibiotic residues in the chicken for tetracycline. The concentration of residues of tetracycline, which were positive in rapid test kit was quantified by high-performance liquid chromatography equipped with a UV detector (HPLC-UV). One muscle sample and one liver sample of chicken have found the concentration of tetracycline as 229 and 339 μg/kg, respectively while chlortetracycline was not detected. This result confirmed widespread misuses of antibiotics especially tetracycline in farm and lack of application of recommended withdrawal times. Antimicrobial assay of chicken extracts was also performed by using the disc-diffusion agar method. The plates were incubated overnight at 37 °C for 18-24 hours, but microbial growth was not observed in all the samples tested.

Keywords: Antibiotic residues; Chicken; HPLC-UV; Quicking Tetracycline rapid Test kit; Tetracycline

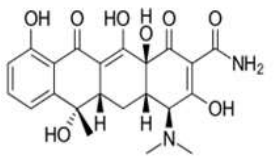
Introduction

A broiler is grown commercially for meat as they grow rapidly and the financial investment is low (Saxena *et al.*, 2014). As the demand for meat is increasing day by day, several agents have been using for growth promotion and treatment of the product. When antibiotics are given to animals, part of the antibiotic and its by-products will result in the flesh and the product (honey, milk, eggs, etc.) of the animal. However, after some days, the level gradually declines because of metabolic degradation. The antibiotic withdrawal period is the time that passes between the last doses of an antibiotic given to the animal and the time when the level of antibiotic residues, *i.e.* animal's flesh or products, falls below the maximum residues limit (MRL). Until the withdrawal period has expired, the animal or its products are not suitable for human consumption (Farm Animals, 2012).

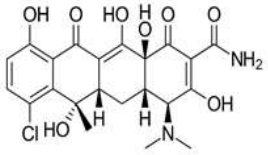
Antibiotics are invaluable chemical probes, mostly produced by soil microbes that inhibit the growth of microorganisms or destroy them. Veterinary medicine utilizes antibiotics as a therapeutic, prophylactic, and growth-promoting agent as well as nutritive purposes in poultry and livestock production. They can be administered either in feed, in drinking water, or by injection, and some

use ‘‘cocktails’’, i.e. mixtures of small amounts of several substances. Residues or their metabolites may cause adverse effects after its consumption to the humans. Carcinogenicity, mutagenicity, bone marrow toxicity (Chloramphenicol) and allergy (Penicillin) are some of the hazards of consumption of antibiotics residues in food (Nisha, 2008; Yu *et al*., 2011). Bacterial resistance is another serious issue due to the disruption of intestinal microflora (Singh *et al*., 2014).

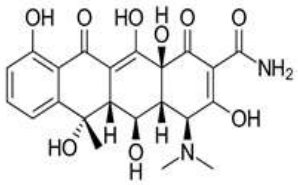
Tetracyclines are a family of antimicrobials that inhibit protein synthesis by preventing attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site (Chopra *et al*., 2001). Tetracyclines consist of a common four-ring structure to which a variety of side chains are attached (Prescott *et al*., 2002). Because of their broad-spectrum activity and low cost, tetracycline (TC) including oxytetracycline (OTC), chlortetracycline (CTC), and doxycycline (DC) are widely used in animals for both prevention, treatment and as feed, additives to promote growth (Abbasi *et al*., 2011).



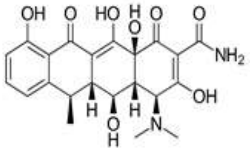
Tetracycline



Chlortetracycline



Oxytetracycline



Doxycycline

Maximum residues limits (MRLs) for TCs were recommended by many agencies. The joint FAO/WHO Expert Committee on Food Additives recommended 200 and 600 µg/kg for chicken muscle and liver, respectively, expressed as the sum of TCs (FAO/WHO report). In addition, the EU had set the MRLs of TCs to be 100 and 300µg/kg for chicken muscle and liver, respectively (*Commission Regulation (EU) No 37/2010*, 2009).

Antibiotic residues are detected by chemical, biological and immunological methods. Detection methods can be classified into qualitative, semi-quantitative, and quantitative. In this study, tetracycline rapid test kit was used to detect the presence of tetracycline residues in animal tissue, and HPLC was used to evaluate tetracycline residues in positive samples resulted from a rapid test kit. Antimicrobial assay of chicken extracts was performed through the disc diffusion agar method.

Materials and Methods

Sample Collection and Preservation (Procedure)

Twenty chicken samples were collected randomly from different meat shops of Kathmandu valley. In most of the previous studies, the antibiotics residues were evaluated in muscle, liver, and kidney (Naeem *et al*., 2006). Hence chicken muscle and liver as samples were purchased and fat tissues were removed. Samples were minced and packed in good quality polybags, sealed and kept in a deep freezer at -20 ℃ till tests were done.

Evaluation of Antibiotic Residues

In this study, two methods were used simultaneously for the determination of antibiotic residues in chicken.

A. Qualitative and semi-quantitative analysis of antibiotic residues by Quicking Tetracycline Rapid Test kit (obtained from Quicking Biotech Co., Ltd. Shanghai, China).
B. Quantitative analysis of meat samples by HPLC equipped with a UV Detector (HPLC-UV).

A. Tetracycline Rapid Test Kit

Principle of the assay

This test is based on competitive lateral flow immunochromatographic assay. The TC-conjugate in the test zone will capture the immune-gold (colloid gold-TC antibody conjugate) when there is very little dissociative TC in the samples. A visible red test band specifies a negative result when the control line (C zone) shows that the card is valid. The test band (T zone) will be not visible if TC is present in a concentration of 100 ppb and above, which explains a positive result. The protocol of the test kit is to be followed as per the instructions are given in the manual provided by the Quicking Biotech Co. Ltd (Tetracycline Rapid Test Kit (tissue), 2010).

a) Equipment for Rapid test kit:

Quicking Tetracycline Rapid Test Kit, Homogenizer, Water bath (80 ℃), Centrifuge, Balance were used.

b) Test procedure

The sample was homogenized at 10,000 rpm for 1 min. 4 g of sample was weighed into 15 mL centrifugal tube, and 0.5 mL of assay buffer was added, and the lid was covered tightly. The tube was put into the water bath (80 ℃) for 10 min, and the extract was taken out as much into 1.5 mL of the centrifugal tube.

Then it was centrifuged at 4000 rpm for 1 min to make extract clear. 0.2 mL of the extract was sucked into microwell with a pipette. Repeatedly sucked, and the sample was extruded until all red reagents were completely dissolved and waited for 1 min. Gradually three drops of sample extraction were dripped into the sample hole “S” of the Cassette horizontally. The result was interpreted in 5-10

min. The result after 10 min. was only considered as reference.

B. Quantitative estimation from High-Performance Liquid Chromatography

Tetracycline was extracted from poultry tissue with Mcllvaine-EDTA buffer having pH 4. The filtered extract was cleaned up and enriched with solid-phase extraction (SPE). Tetracycline was separated by liquid chromatography using a C18 column and measured with a UV detector at 350 nm. The signal of the sample was compared with those from standard solutions of Tetracycline, and Chlortetracycline. Concentration in the sample was calculated with the help of the formula reported (Macneil *et al*. 1996).

Sample processing equipment and Devices and Consumables for HPLC

i. **Apparatus:** The HPLC equipped with a UV detector was used. Analytical balance, pH meter, Centrifuge, Sonicator, Vortex mixture, Shaker, SPE aperture, C18 SPE cartridges, and Nitrogen evaporation device were used for sample preparation.

ii. **Chemicals and reagents:** Standard NIST traceable CRM of tetracycline & chlorotetracycline were used. Methanol, water, and formic acid were of HPLC grade. Na_2HPO_4, EDTA dihydrate, citric acid monohydrate, and oxalic acid dehydrate were of analytical grade.

b) Extraction Mixture

Mcllvaine Buffer: Na_2HPO_4 (28.4 g) was dissolved in 1 L volumetric flask with water, and 21 g citric acid monohydrate was dissolved in 1 Lvolumetric flask with water. The citric acid solution (500 mL) and 312.5 mL Na_2HPO_4 solution were combined, and pH was adjusted to 4±0.05 by using either 0.1 M HCl or 0.1 M NaOH solution.

Na_2EDTA-Mcllvaine Buffer: $Na_2EDTA{\cdot}2H_2O$ (30.25 g) was added to the previously prepared Mcllvaine buffer solution and dissolved.

Methanolic oxalic acid: Oxalic acid (1.26 g) was weighed and dehydrated into 1 L volumetric flask and dissolved in methanol, and volume was made up to the mark with methanol.

c) Preparation of Mobile Phase

Mobile phase A: Oxalic acid (1.26 g) was dehydrated into a 1 L volumetric flask, dissolved in water and made volume up to the mark with water. It was filtered and sonicated.

Mobile phase B: Acetonitrile (600 mL) and 200 mL methanol were added in a beaker and sonicated.

d) Standard Solutions

Stock standard solutions of each standard (tetracycline and chlortetracycline) was prepared by dissolving 10 mg of the compound in 10 mL methanol to obtain a final concentration of 1000 ppm. Stock standard solutions were diluted with methanol to give a series of working standard solutions that were prepared frequently.

e) Sample Extraction

Sample (5 g) was taken in a 50 mL centrifuge tube, and 20 mL extraction buffer was added. The mixture was then closed with a screw cap and vortexed for 30 seconds followed by 10 minutes shaking in a mechanical shaker. The mixture was centrifuged at 6000 rpm for 10 minutes. The upper layer of the supernatant solution was taken in a New 50 mL centrifuge tube. The extraction steps were repeated twice with 15- and 10-mL extraction buffer respectively. Supernatants were collected at each extraction step, and all supernatants were mixed into the same centrifuge tube, then centrifuged for 15 min at 6000 rpm. A filter paper was placed in a funnel and moistened with 1 mL extraction Buffer. The supernatants were filtered. The centrifuge tube was washed and filtered with 4 mL of extraction buffer. Centrifuged for 15 min at 6000 rpm and proceed with SPE.

f) SPE Cleanup

Conditioning: The SPE cartridges conditioned with3 times 2 mL MeOH under gravity flow and discarded the eluate. Next, three times 2 mL of water was added with gravity flow and discarded the eluate (Precaution: cartridges should not be run in dry).

Loading: The extract was added stepwise into the cartridge with a gentle vacuum (speed approx. 1-2 drop/s). Cartridges should not run dry during loading.

Drying: Cartridges was dried for 2 min using a vacuum.

Elute: The contents of cartridges were eluted using 3 times 2 mL Methanolic oxalic acid into 10 mL volumetric flask.

After elution, volume was made up to the mark with water and shaken. Filtered through 0.45µm syringe membrane; filtered into HPLC vial and injected into the HPLC system.

g) Detection and Quantification

The separated TCs were detected with Ultraviolet detector, and the quantification was integrated by chromatographic software interfaced to a personal computer and using the regression equation.

Antimicrobial Activity

a. **Antimicrobial activity assays:** Antimicrobial assay of chicken extracts was performed by using Nutrient Agar(NA) Medium. Nutrient Broth is convenient, as most bacteria grow in this type of medium.

b. **Preparation of extracts:** Meat sample was homogenized at 10,000 rpm for 1 min. 4 g of it was weighed into 15 mL centrifugal tube, and 500 µL of 50 mM Tris buffer (pH 8) was added. Then centrifuged at 12,000 rpm for 1 min to make the extract clear. The supernatant was used for the assays.

c. **Screening and Evaluation of Antimicrobial Activity:** All instruments, solutions, and media before using them for plating procedures were sterilized, and workspace was set up in a Biosafety cabinet.

The supernatant was picked up and spread over the surface of the NA agar medium using a rapid, smooth, back-and-forth motion by a metal loop. The plate was inverted and set down back into the lid. The plates were incubated overnight at 37 °C. After proper incubation (18-24 hours), the plates were observed for bacterial growth.

Results and Discussion

Antibiotic Residues in Chicken Analyzed by Rapid Screening Test Kits

Out of 20 meat samples tested by Quicking Tetracycline Rapid Test Kit, two samples were found positive for the tetracycline residues (shown in **Fig. 1**). Semi-quantitative tests revealed that all positive samples contained tetracycline residues in the concentration of 100 ppb and above (**Table 1**). The results were interpreted based on the clear band appearing in the C and T zone.

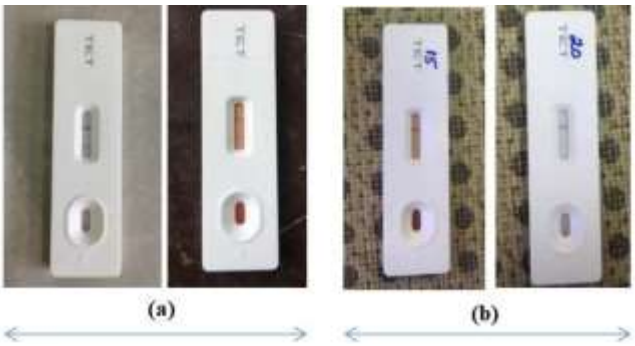


Fig. 1: Samples showing (a) the negative and (b) positive results for Tetracycline residues.

In **Fig. 1 (a)** the result showed that tetracycline residues were absent in a meat sample due to the appearance of the clear band in both C and T zone while in **Fig. 1 (b)** the result showed that tetracycline residues were present in the meat sample as the clear band appeared in only C zone.

Antibiotic Residues Analyzed by HPLC Method

Wavelength Screening: In this study, 350 nm was used for the tetracycline absorption wavelength with a UV detector.

Linearity and Range: Standard tetracycline solution of five different concentrations ranging from 50 to 1000 ppb was analyzed. The calibration curve was established according to the peak area and the concentration of the Tetracycline in standard solutions. The result showed a linear relationship in the concentration range of 50 to 1000 ppb (shown in **Fig. 2**).

Table 1: Tetracycline residues in chicken samples from Kathmandu valley analyzed by Quicking Tetracycline Rapid Test Kit.

S. No.	Sample Type	Location	Tetracycline
1	Muscle	Sanepa	Negative
2	Liver	Sanepa	Negative
3	Muscle	Kalimati	Negative
4	Muscle	Kalanki	Negative
5	Muscle	Lagankhel	Negative
6	Muscle	Balaju	Negative
7	Muscle	Bhaktapur	Negative
8	Muscle	ChappalKarkhana	Negative
9	Muscle	Basbari	Negative
10	Muscle	Maharajgunj	Negative
11	Muscle	Aakasedhara	Negative
12	Liver	Teku	Negative
13	Muscle	Kirtipur	Negative
14	Liver	Godawari	Negative
15	**Liver**	**Khokana**	**Positive**
16	Muscle	Khokana	Negative
17	Liver	Harisiddhi	Negative
18	Muscle	Patan	Negative
19	Muscle	Samakhusi	Negative
20	**Muscle**	**Tokha**	**Positive**

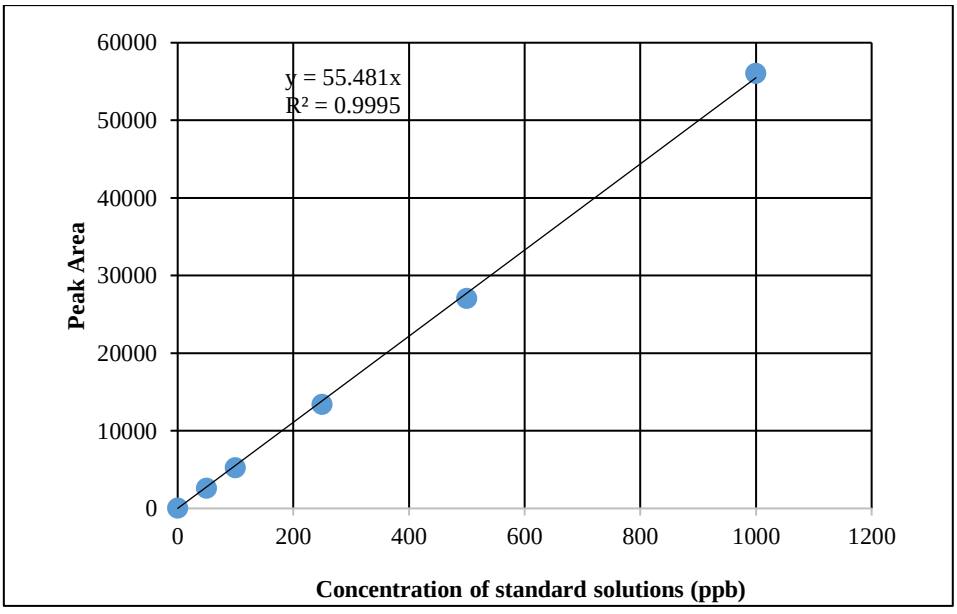


Fig. 2: Calibration curve for the standard Tetracycline solutions

Sensitivity

Limit of Detection

The limit of detection is used to decide whether an analyte is present, while the limit of quantification is used to decide whether the concentration of an analyte can be reliably determined (Michaele *et al*., 1999). Mathematically, it can be expressed as:

$$Limit\ of\ detection = \frac{3 \times standard\ deviation\ of\ intercept}{slope\ of\ calibration\ line} \quad \ldots\ldots\ldots \quad (1)$$

Limit of Quantification

Limit of quantificationis the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of the test (Shrivastava et al., 2011). Mathematically,

$$Limit\ of\ quantification = \frac{10 \times standard\ deviation\ of\ intercept}{slope\ of\ calibration\ line} \quad \ldots\ldots\ldots \quad (2)$$

The limit of detection and limit of quantification were 30 ppb and 100 ppb, respectively. The regression equation for tetracycline was:

$$y = 55.481x \quad \ldots\ldots\ldots \quad (3)$$

The coefficient of determination (R^2) of the line was 0.9995.

All the results obtained from this experiment are presented in the following assay validation Table 2.

Table 2: Assay validation Table

S. No.	Parameter	Value
1	Accuracy (mean ± s.d) (in %)	96.278 ± 3.212
2	slope (a)	55.481
3	Linearity range (in ppb)	50: 1000
4	Coefficient of determination (R^2)	0.9995
5	The standard error of intercept	285.005
6	The standard deviation of the intercept	635.562
7	Limit of detection (in ppb)	30
8	Limit of quantification (in ppb)	100

Results of Tetracycline Residues in Chicken Sample

Tetracycline residues in the chicken samples that were sold in the market of Kathmandu and were positive inQuicking Tetracycline Rapid Test Kitwere analyzed by using HPLC coupled to ultraviolet detection. Peak identification of the sample was based on the comparison with a retention time of standard compounds peaks. The external standard method was used for quantification using calibration curves fitted by linear regression analysis. The chromatograms obtained from the highest standard solution of Tetracycline and samples of meat are shown in **Fig. 3** respectively. [In all the chromatograms the X-axis is retention time in minutes and Y-axis is Detector response in milli-Absorbance Units (mAU)].

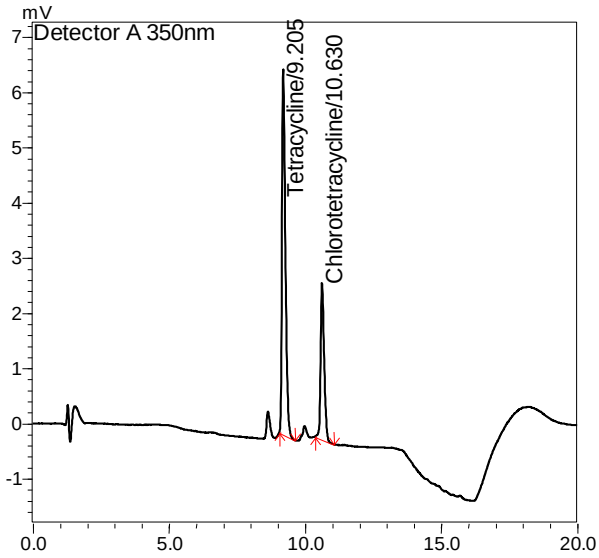


Fig. 3: HPLC Chromatogram of 1000 ppb STD (Highest STD)

From the chromatogram of standard Tetracycline solution, it has been shown that the retention time for Tetracycline was at 9.205 minutes. This retention time was used for the identification of the peak of interest in the chromatogram of the sample. The retention time for chlortetracycline was 10.630 minutes.

Tetracycline residues were detected in chicken samples, which were identified based on retention time, *i.e.* 9.205 minutes, while no detection level signal was seen for the retention time 10.630 minutes and hence tetracycline's analytical signal was only detected and is shown in **Fig. 4**.

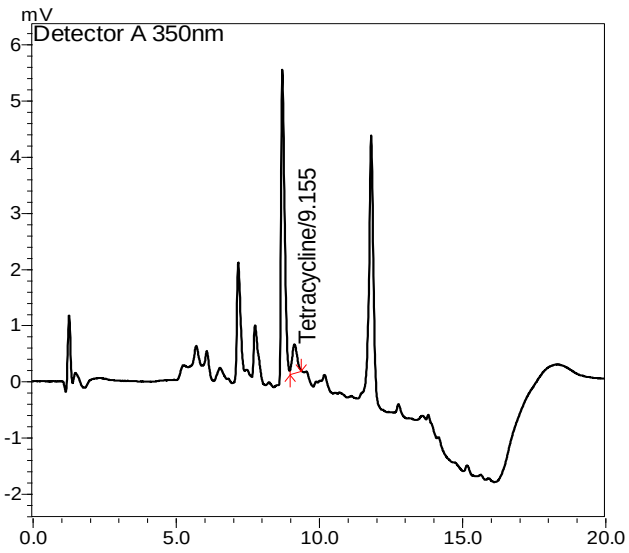


Fig. 4: HPLC Chromatogram of chicken liver (171.97 ppb in aliquot).

By knowing the value of the analytical signal of the unknown from the HPLC, the concentration of tetracycline in HPLC was calculated by using the following formula:

$$Concentration\ of\ Tetracycline\ in\ HPLC = \frac{Analytical\ signal - intercept}{slope} \quad \ldots\ldots \quad (4)$$

The concentration of tetracycline residues in aliquot (HPLC) of liver and muscle samples was found to be 171.97 ppb and 115.08 ppb, respectively.

Then the concentration of tetracycline obtained in the sample was calculated by using the following formula:

$$Conc.of\ Tetracycline\ in\ poultry\ tissue(\mu g/kg) = \frac{HPLC\ reading\ \ (ppb) \times Final\ volume\ in\ HPLC}{weight\ taken\ (g)} \ldots (5)$$

The quantity of tetracycline in liver and muscle was 339 and 229 µg/kg, respectively, and is shown in **Fig. 5**.

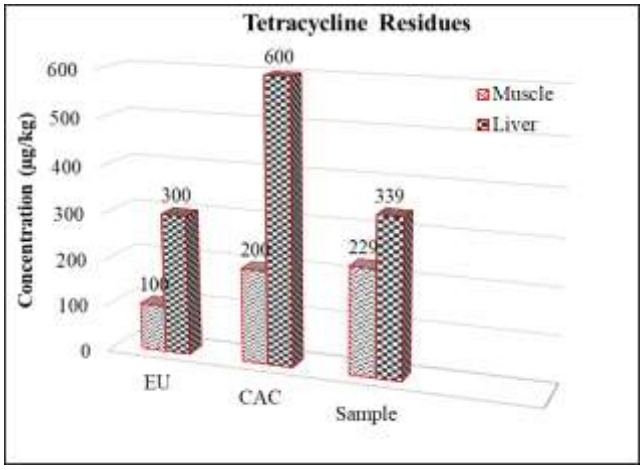


Fig. 5: Concentration of Tetracycline residues [*EU and CAC are MRL values of Tetracycline Residues according to European Union and Codex Alimentarius Commission respectively, while a sample is tetracycline residues of the present studied sample.]

The quantitative value of tetracycline residue in the muscle of the studied sample was found higher than the MRL value of both EU and CAC while that of the liver's tetracycline residue value was higher than EU's but within CAC's maximum residue limit.

This preliminary research was reported the presence of antibiotic residues in poultry samples coming Kathmandu. Among the primary screening of the 20 samples of chicken meat samples, 2 samples were found contaminated with relatively high tetracycline residue. The level of antibiotic consumption in the Nepalese veterinary medicine should be evaluated.

Antimicrobial Screening

Antimicrobial assay of chicken extracts was performed by using Nutrient Broth Agar Medium. The plates were incubated overnight at 37 °C. After proper incubation (18-24 hours), bacterial growth was not noticed in all the samples (data not shown).

Conclusion

Residues of antibiotics were detected in the chicken of Kathmandu by Rapid screening test kit and HPLC. Results of the tetracycline Rapid Screening Test Kit showed that two samples were positive for tetracycline residues. The positive samples were further quantified by HPLC. The liver and muscle samples were found to contain 339 and 229 µg/kg tetracycline residues, respectively. Chlortetracycline was not detected in both samples.

This study urges the need of an intervention to decrease the level of antibiotics residues in chicken samples through launching educational and awareness programs on the prudent use of antibiotics in animal husbandry. The studies should be conducted to screen a larger sample collected from different farms located in different regions in the country. The subsequent researches would need focus on various parts of Nepal to complete the available data and to provide a complete view of antibiotic residues in chickens for entire country.

Author's Contribution

VK Jha designed the research plan; S Patrabansh performed experimental works, collected the required data & prepared the manuscript. S Patrabansh, N Parajuli, and VK Jha analysed the data; critically revised and finalized the manuscript. Final form of manuscript was approved by all authors.

Conflict of Interest

The authors declare that there is no conflict of interest with present publication.

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