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ABSTRACT

BACKGROUND: Antibiotics are among the most widely used drugs in farm animals for therapeutic and prophylactic purposes. However, the antibiotic residues in meat are a serious public health concern due to their harmful effects on consumer health. Besides this problem, there is limited information on the level of the antibiotic residues, including tetracycline residues. Therefore, this study was aimed to determine the tetracycline residues in the kidney and muscle samples of beef cattle in Harar town and Dire Dawa city.

METHODS: A study was conducted on 500 randomly selected carcass (250 kidney and 250 muscle samples) slaughtered at Dire Dawa and Harar municipal slaughterhouses between December 2018 and December 2019. The samples were collected aseptically and screened for tetracycline residues by thin layer chromatography. Then, presumptive positive samples were analyzed using high-performance liquid chromatography to get a quantitative outcome. Descriptive statistics were used to determine the frequency, mean, or standard deviation to determine the summary values and distribution of the outcomes. Finally, the data was analyzed using SPSS version 21 software.

RESULTS: Out of 500 samples, oxytetracycline residues were detected in 84% of the samples. However, tetracycline and doxycycline were not detected in all samples. Among the kidney and muscle samples collected from Dire Dawa and Harar abattoirs, 109 (87.2%) and 101 (80.8%) were positive for oxytetracycline, respectively. Oxytetracycline residue levels in Dire Dawa ranged from 57 to 607 µg/kg for the kidney and 10.14 to 435 µg/kg for muscle samples. Among the samples collected from Harar, the concentration of oxytetracycline residues ranged from 16 to 433 µg/kg and 6 to 435 µg/kg for kidney and muscle samples, respectively, at Harar slaughterhouses. About 22.0% of muscle samples collected from Dire Dawa and 17.8% from Harar town had oxytetracycline residues above maximum residue limits.

CONCLUSIONS: In general, the study revealed that oxytetracycline residues were prevalent among tetracycline residues analyzed from kidney and muscle samples in the study areas. Thus, there is a risk of consumer exposure to these antibiotic residues that may have human health effects. Therefore, awareness creation and strict regulation is needed by the regulatory authorities for the use of antimicrobial drugs in the livestock industry.

KEYWORDS: Antibiotic residue, abattoirs, HPLC, TLC, beef, kidney, muscle, tetracycline, meat

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Introduction

Food safety is of great importance to consumers' health. Currently, hundreds of substances are used as growth enhancers, prophylaxis, and treatment for diseases in food-producing animals.[1,2] Antibiotics are among the most widely used veterinary drugs for therapeutic purposes.[3] Among the veterinary antibiotics, tetracyclines (TCs) are antibacterial substances that are commonly used in both human and veterinary practices.[4]

According to the Food and Drug Administration (FDA), TCs show the highest level of drug use in food producing animals.[5] They are widely used for the prevention and treatment of diseases as well as feed additives to promote growth,[1,3,6] and may lead to residues appearing in meat and eggs.[1,6] Tetracyclines are antibiotics that include tetracycline, oxytetracycline, chlortetracycline, doxycycline, and minocycline.[7-9]

The beneficial therapeutic properties of TCs, a broad spectrum of antibacterial activity, and price considerations make them widely used in veterinary practice.[4] However, the extensive use of antibiotics for purposes other than therapeutic purposes has led to the presence of antibiotic residues in animal originated food products and the development of bacterial drug resistance.[3,10] They have the potential to contaminate foods of animal origin.[11]

For example, beyond a maximum residue level (200 µg/kg in muscle and 1200 µg/kg in kidney) TCs residues in animal products has adverse health effects.[12] Their residues in foods, especially in meats, may lead to several adverse health effects, such as the development of multi-drug resistant microbial strains.[11,13,14] It has many consequences, such as increasing morbidity (pose toxic and dangerous effects on the consumer's

health; allergic reactions, poor fetal development, and gastrointestinal effects) and mortality rates, as well as increasing hospital stays, and leading to an increase in medical costs.[7,15-17]

The use of antimicrobial agents in food-producing animals has become a public health concern, especially in developing countries where such drugs are administered indiscriminately.[18-20] In many developing countries, including Ethiopia, where there is a scarcity of veterinary professionals and a lack of enforcement of regulations, the antibiotics are administered indiscriminately. This makes the control of antibiotic residues an important measure to protect consumers.[15,21]

Besides these problems, the control of drugs from the government authorities and information on the actual rational drug use are limited in Ethiopia.[18] Furthermore, there is a lack of awareness and preparedness in dealing with the risk of indiscriminate use of antimicrobials in Ethiopia.[18] Food animals slaughtered for domestic and export purposes in the country are not screened for the presence of residues in any of the slaughterhouses in the country that aim to protect the consumers' health.[18] Only a few studies conducted in Ethiopia provide the information on the level of antibiotics residues.[18,22]

Therefore, the current study aimed to detect and determine the residue levels of tetracyclines in the kidney and muscle of beef cattle slaughtered at Dire Dawa and Harari Municipal Abattoirs, in Eastern Ethiopia.

Materials and Methods

Description of study area and study design

A Cross-sectional study was conducted in Dire Dawa City and Harar Town Abattoirs from December 2018 to December 2019. The Harari region is divided into 9 districts and 36 lower administrative units (kebeles). The region is located at 526 km east of Addis Abeba at a latitude of 8°50′ to 9°15′N and a longitude of 9°36′N, 41°52′E, at an elevation of 1850 m above sea level.[23]

Dire Dawa administration is located at about 515 km to the east of Addis Ababa. The area is found between 9°27′ and 9°49′N latitude and 41°38′ and 42°19′E longitude. Dire Dawa administration is divided into 9 urban kebeles (lower administrative units) and 32 peasant associations. The mean annual rainfall of the area varies from 550 mm in the lowland northern part to 850 mm in the southern mountains, with an average of 640 mm.[23] The map of the study location is provided below (Figure 1).

Sample size determination

The sample size for each study location was calculated by taking into account the prevalence of tetracycline residues reported by a previous study that reported 82.1% of samples

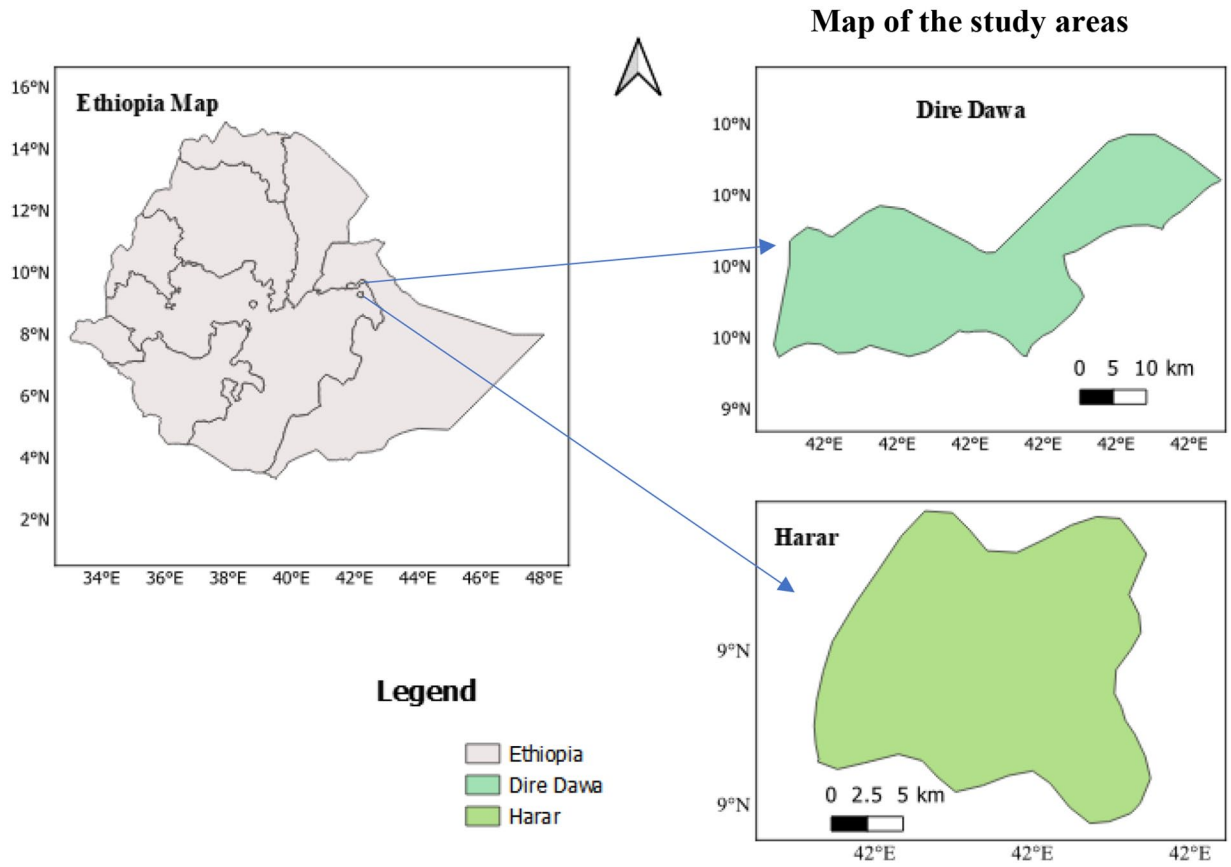


Figure 1. Map shows study areas, Harar town and Dire Dawa city, eastern Hararghe, Ethiopia.

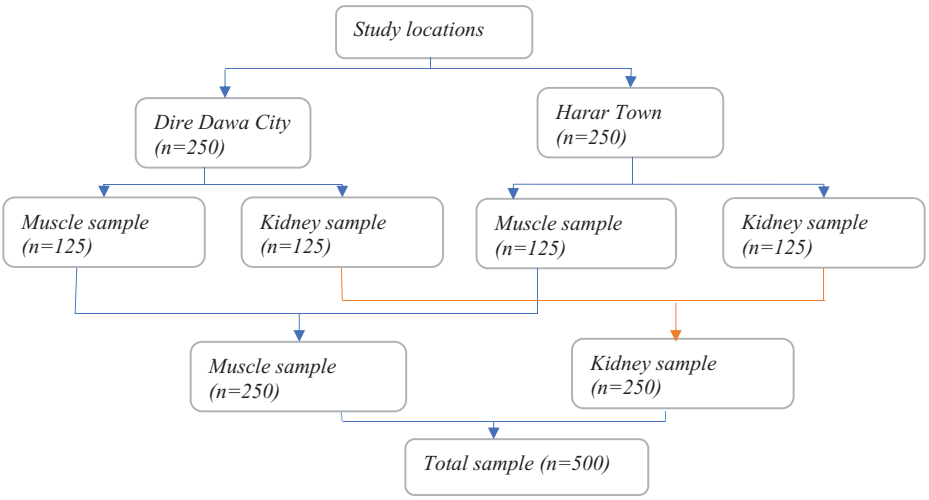


Figure 2. Sampling technique used to select required sample size for the current study (n = 500).

had tetracycline residues.[18] Then, the sample size required for the current study was calculated using the formula below by considering 95% confidence levels, a 5% degree of precision, and 10% for uncertainty.

$$n = Z\alpha / 2^2 \frac{p(1-p)}{d^2}$$

Where, n = sample size required.
$Z_{\alpha/2}$ is the value of the standard score at 95% confidence interval (1.96); the assumptions of confidence level are at 95% = 1.96.
P = estimated prevalence.
d^2 = degree of precision.

Finally, 500 samples (250 kidney and 250 muscle samples) were collected from 125 carcass of animals.

Sampling methods and techniques

A total of 500 samples (250 kidney and 250 muscle samples) were randomly collected from the selected animals to determine tetracycline residues in kidney and muscle in Dire Dawa and Harar Municipal Abattoirs. A random sampling method was used to select the required sample size for the current study.[18] Each beef cattle fit for slaughter was registered to select the required sample size from healthy beef screened for slaughter. The regulatory bodies determined the fitness of beef for slaughter. Finally, a total of 125 kidney and 125 muscle samples were collected from each location (Figure 2).

Sample collection and processing

After the animals were selected, 100 g of tissue samples from each kidney and muscle were aseptically collected after slaughter in a separate sterile sample falcon tube, from each animal recruited to the study. To get the composite samples, particularly for muscle sample, the samples were taken from different sites (different types of muscles). The samples were collected within a range of 30 minutes to 1 hour after slaughter. Then, the samples were transported to Haramaya University Laboratory, packed in an icebox and stored at a temperature of −18 to −20°C until they were further transported for analysis, drug administration and quality control authority laboratory.[24] Then, the samples were analyzed at the Ethiopian drug administration and quality control authority laboratory, Addis Ababa.

Sample preparation. As per a previous study, the muscles were chopped and kept frozen (−20°C) until the analysis time. Frozen samples were thawed. Two grams of each sample was weighed into a polypropylene tube after it was cut into small pieces and homogenized, and then 0.8 mL of 20% trifluoroacetic acid and 0.4 mL of 0.01 M EDTA were added.[7,25] Then, the extracts were heated in a water bath at 54°C to inactivate the complement system and other natural antimicrobial systems. The mixture was vortexed for 2 minutes. All separations was performed under gradient elution with mobile phase composed with a mixture of methanol:acetonitrile:0.03M oxalic acid buffer:water, which kept isocratic mode. Then, mixture of methanol:0.01M citrate was added to obtain a total volume of 5 mL. Then, the mixture was vortexed for 2 minutes and sonicated for 10 minutes at room temperature. After that, it was centrifuged at 4000 rpm for 20 minutes. The supernatant was filtered through a 0.22 µm nylon filter and injected into the HPLC for analysis.[7]

Tetracycline residue analysis methods

In the current study, tetracycline residues in kidney and muscle were detected using chromatographic analysis.[18] Tetracycline residues in kidney and muscle samples were identified using thin-layer chromatography (TLC). Furthermore, tetracycline residues were analyzed using high performance liquid chromatography (HPLC) for quantification as per manufacturer protocol. Tetracycline residues were detected using UV detector at wavelength set at 355 nm. High-performance liquid chromatography (HPLC) in the reverse-phase mode, with different

detection modes, is commonly used to determine veterinary antibiotics.[4,26]

Detection of tetracycline residues using TLC. Thin Layer Chromatography (TLC) was used for the detection of tetracycline residues following the recommended techniques.[27,28] Thin layer chromatography is a sensitive and exact method for monitoring low amounts of different biological and chemicals. Illumination of tetracycline against UV light helps as a simple detector for the analysis. To extract tetracycline, the sample was prepared using 10 g of tissues of cattle in 10 mL of 96% ethanol. Then, it was crashed and squeezed fine in a Chinese mortar. The solvent was transferred into 15 mL falcon centrifuge tubes and centrifuged at 4000 rpm for 10 minutes. The clear supernatant was transferred to clean glass test tubes and evaporated in water bath at 80°C. After full drying, the deposits resolved in 0.2 mL methanol.[27]

To prepare silica plates, glass plates (10 × 20 cm dimensions) were washed in acetone bath. For each plate 2 g of silica gel 60 plates (Merck, Germany) mixed in 5 mL distilled water and mixed thoroughly to produce fine paste. Clean glass plates were coated with silica paste by TLC gel spreader system (CAMAG, USA) in 0.25 mm thickness. Plates were activated in 120°C for 2 hours. The pH of disodium EDTA was adjusted to 7.0 by a 10% w/v solution of 10 molar sodium hydroxide and sprayed evenly onto the plates (about 10 mL for a plate of 100 × 200 mm). The plates were then allowed to dry horizontally for at least 1 hour.[27,29]

Furthermore, the standard was prepared for the comparison of extracted residues with antibiotics, reference standard (Sigma Chemical Co., St. Louis MO, USA) supplied analytical standards of oxytetracycline, tetracycline, and doxycycline of 1 µg/mL dissolved in methanol.[30] For pointing, running and detection, 10 mL of methanol dissolved deposits were pointed on silica plates and treated plates transferred to TLC tank containing a saturated mixture of dichloromethane, methanol and water as mobile phase. After receiving of solvent front to end of plates they were removed off and dried in a current air and examined under ultraviolet light at 366 nm.[31,32]

Determination of tetracycline residue levels using HPLC. Samples that were positive in thin layer chromatography was subsequently analyzed using high performance liquid chromatography following the techniques recommended by Agence Franaise de Securite sanitarie des aliments[33] and protocol[4] for determination of tetracycline residues using HPLC. Determination of the tetracycline residues was done using a high-pressure liquid chromatography (model Shimadzu Class-VP Series, Kyoto, Japan) equipped with SIL-10 auto-injector with sample cooler and LC-10 on-line vacuum degassing solvent delivery unit.

Chromatographic control, data collection, and processing were carried out using Shimadzu Class VP data software, a constant flow pump and a variation wavelength UV detector set at 355 nm was used for analyzing the data. The separation was performed on a Nucleosil C18 (5 µm, 250 × 4.0 mm I.D.E Merck) column with acetonitrile and 0.01 M aqueous oxalic acid solution by gradient mode as the mobile phase flow-rate of 0.8 mL/min at room temperature and the sensitivity range was 0.08 ppm.

Data management and analysis

The data was entered into databases using Micro-Soft Excel 2010 computer program analysis and SPSS version 21. Descriptive statistics such as percentage and frequency distributions were used to describe the nature and characteristics of the data. However, Statgraphics version 5.0 (Windows, www.Statgraphics.com) via the Duncan Multiple Comparison Test enabled the comparison of the concentration of the maximum residue limit of kidney and muscle of beef cattle.

Results

Control samples

The non-spiked samples from the control animals peaked at a different time from that of the analytical standards, whereas both oxytetracycline-spiked samples peaked at 10 minutes as expected. The higher concentration of oxytetracycline was associated with a higher peak. The peak of extracted oxytetracycline was also detected at 7 minutes and was highly similar to the spiked control sample (Figure 3).

Calibration

Calibration curve depicting the best-fit line from analytical oxytetracycline standards is provided below (Figure 4).

Results of the qualitative analysis using TLC

Analysis of kidney samples with TLC showed that the majority of the samples had different amounts of oxytetracycline residues. At the Dire Dawa slaughterhouse, approximately 87.2% of kidney and liver samples were positive for oxytetracycline residues. Correspondingly, likewise, in the town of Harar, approximately 80.8% of kidney and muscle samples were positive for oxytetracycline residues.

Results of the quantitative analysis using HPLC

The samples positive for TLC analysis were further analyzed using HPLC for quantification, to determine the concentration of tetracycline residues in the samples. Oxytetracycline residues in kidney and muscle ranged from 57 to 607 µg/kg and 10.14 to 435 µg/kg in samples collected at the Dire Dawa slaughterhouse, respectively. The oxytetracycline residue levels in kidney and muscles ranged from 16 to 433 µg/kg and 6 to 435 µg/kg, respectively, in Harar town's abattoir. There was no

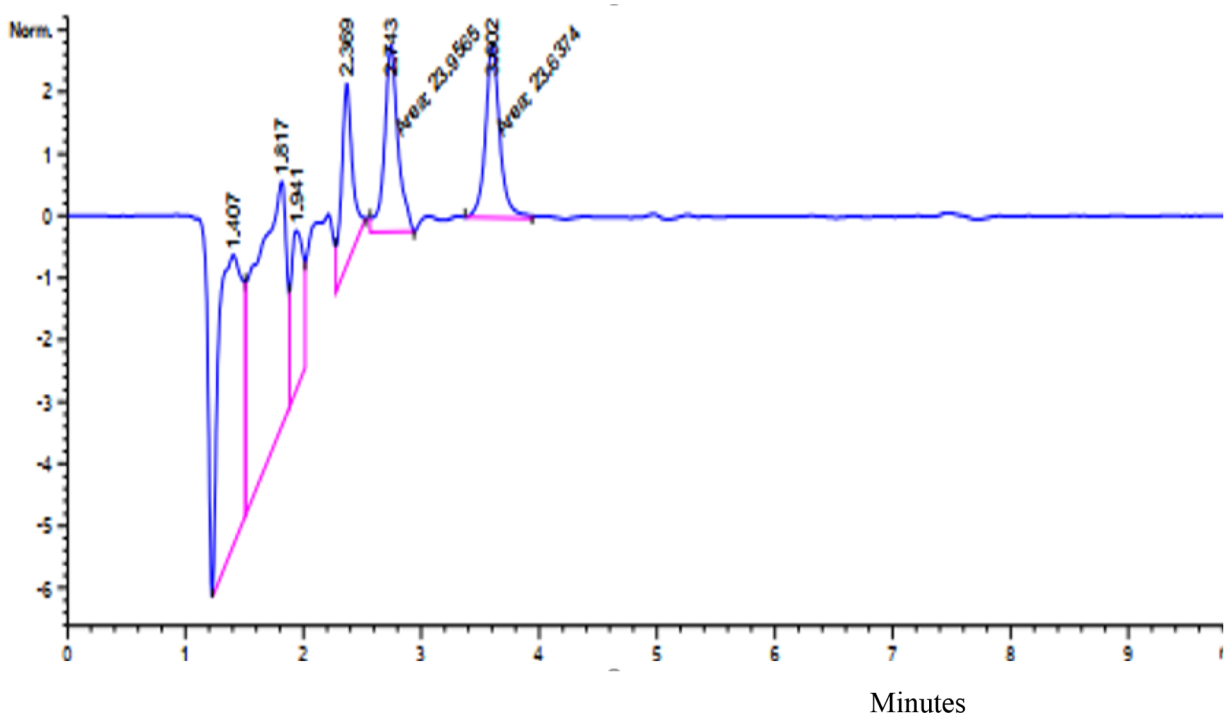


Figure 3. Combined chromatogram of the negative control sample (without oxytetracycline) and the sample spiked with 10 mg/kg 20% oxytetracycline.

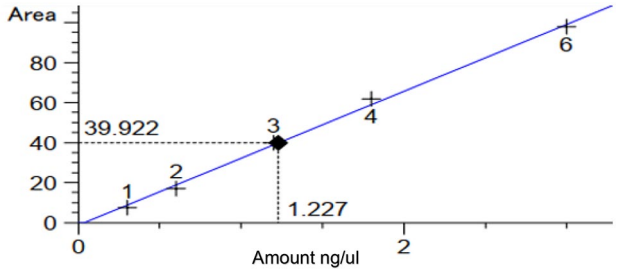


Figure 4. Calibration curve depicting the best-fit line from analytical oxytetracycline standards.

statistically significant (a *P*-value of >0.05) variation between study locations (Table 1).

The current study showed that 22.0% and 17.8% of the muscle samples collected from the Dire Dawa city and Harar town, respectively, exceeded the recommended maximum residue level. However, none of kidney samples exceeded the maximum recommended limit. All kidney samples are within the recommended level (Table 2).

The graphical presentation of mean detectable concentrations (µg/kg) of oxytetracycline in muscle samples in comparison with the maximum residue limit among samples collected from Dire Dawa slaughterhouse (Figure 5).

The graphical presentation of mean detectable concentrations (µg/kg) of oxytetracycline in muscle samples in comparison with the maximum residue limit among samples collected from Harar slaughterhouse (Figure 6).

Furthermore, some results of oxytetracycline residue in analyzed samples are provided below (Figure 7a-g).

Discussion

In this study, 500 samples (250 kidney and 250 muscle samples) were analyzed to detect and determine the level of tetracycline residues. Of 500 samples analyzed, oxytetracycline residue was detected in 84% of the samples. The findings of this study were relatively in line with the finding of another study conducted in Ethiopia that reported about 71.3% of the samples had oxytetracycline residues.[18] *S*imilarly, it was in line with the finding reported from Uganda, which reported that the prevalence of positive samples ranged from 56.88% to 84.52%.[35] Similarly, the finding of the current study was in line with the finding of another study conducted in Iran, which reported 75% of meat samples positive for tetracycline.[36] The findings suggest that oxytetracycline was indiscriminately used in the study areas. Furthermore, it may indicate that the recommended withdrawal time may not have been respected before slaughtering of the animals.

In this study, tetracycline and doxycycline were not detected in all samples, which is consistent with the findings of another study conducted in Ethiopia that reported none of the samples had a detectable tetracycline and doxycycline residues.[18] However, the current findings are lower than those reported in India[37] which found about 18.89% and Tanzania[38] reported 35% of samples positive for oxytetracycline.

Depending on the type of sample, 109 (87.2%) kidney and 101 (80.8%) muscle samples were positive for oxytetracycline. In addition, the current study showed that the mean concentration of oxytetracycline residues in kidney and muscle samples was 131.01 ± 99.9 (SD) and 114.7 ± 98.55 (SD) µg/kg,

Table 1. Overall characteristics of the samples positive for oxytetracycline residues.

CHARACTERISTICS	DIRE DAWA CITY		HARAR TOWN	
	KIDNEY SAMPLES	MUSCLE SAMPLES	KIDNEY SAMPLES	MUSCLE SAMPLES
No. positive (%)	109/125 (87.2)	109/125 (87.2)	101/125 (80. 8)	101/125 (80.8)
Mean (SD)	131.01 ± 99.85	114.7 ± 98.55	127.33 ± 85.92	103.81 ± 89.64
Range	57-607	10.14-435	16-433	6-435

Abbreviation: SD, standard deviation.

Table 2. Number of oxytetracycline containing sample exceeding the maximum recommended level.[34]

TYPES OF SAMPLE	ABATTOIRS	NO. POSITIVE SAMPLE	NO. POSITIVE SAMPLE ABOVE MRL	% OF SAMPLE MRL	MRL (μG/KG)
Muscle	Dire Dawa	109	24	22.0	200
	Harar	101	18	17.82	200
Kidney	Dire Dawa	109	0	0	1200
	Harar	101	0	0	1200

Abbreviation: MRL, maximum residue level; No, number; OTC, oxytetracycline.

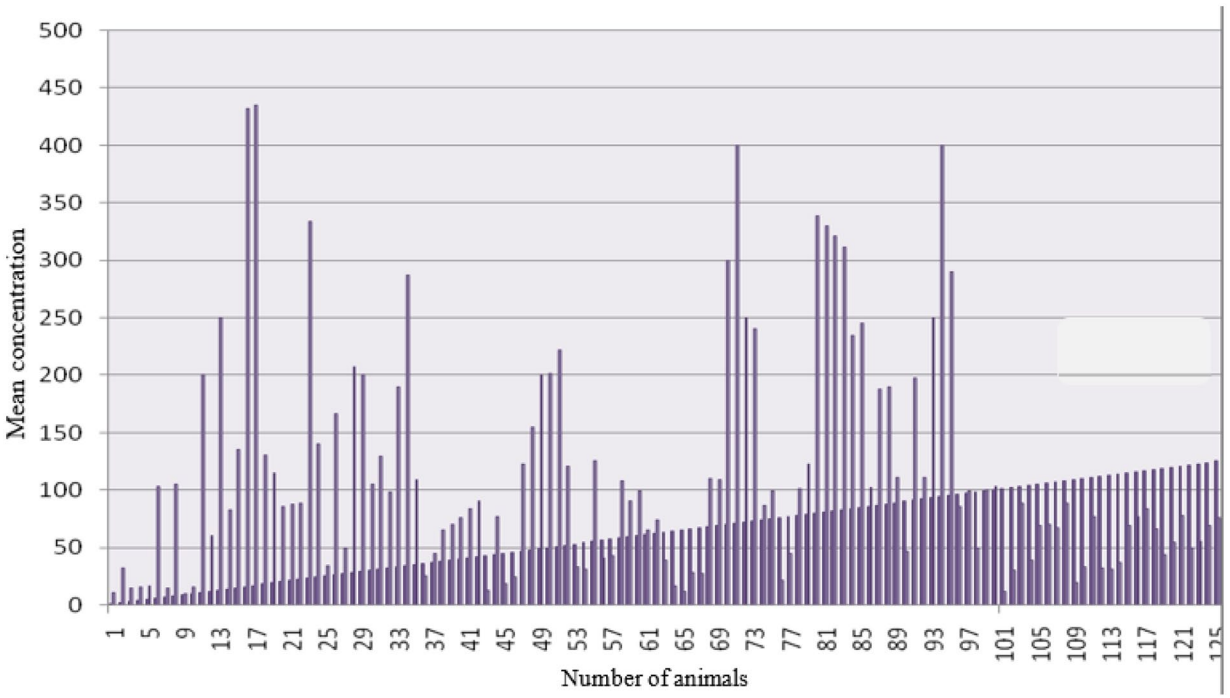


Figure 5. Mean detectable concentrations (μg/kg) of oxytetracycline in muscle samples in comparison with the maximum residue limit among sample collected from Dire Dawa City.

respectively, for the samples collected from the Dire Dawa slaughterhouse. The current study found a higher concentration of oxytetracycline in the muscle samples than the findings reported in other regions of Ethiopia, such as Addis Ababa, Nazareth, and Debre Zeit, where the mean concentration was accounted for at 108.34, 64.85, and 15.916 g/kg, respectively.[18]

Furthermore, the current study found a higher concentration of oxytetracycline in the kidney than another finding reported in Addis Ababa, Nazareth, and Debre Zeit slaughterhouses, which reported 99.02, 109.35, and 112.53 μg/kg in Debre Zeit slaughterhouses, respectively. Similarly, in Harar, the concentrations of oxytetracycline in kidney and muscle samples were 127.33 ± 85.92 and 103.81 ± 89.64 μg/kg,

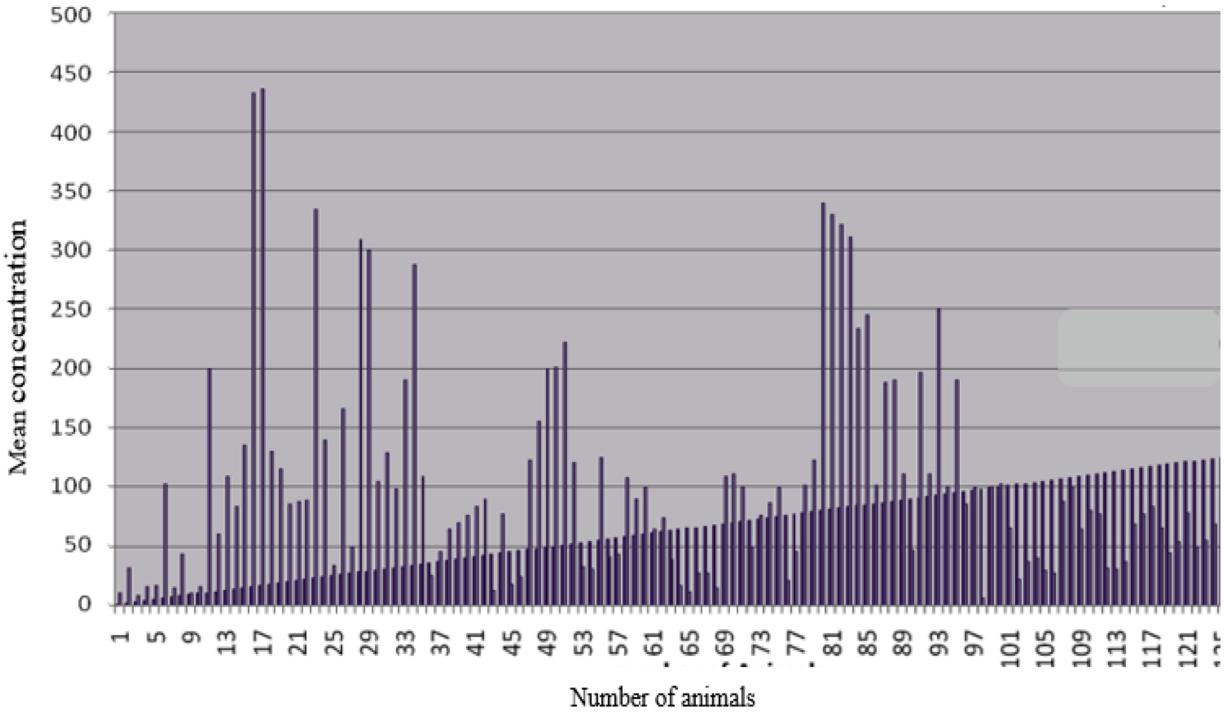


Figure 6. Mean detectable concentrations (µg/kg) of oxytetracycline in muscle samples in comparison with the maximum residue limit among sample collected from Harar Town.

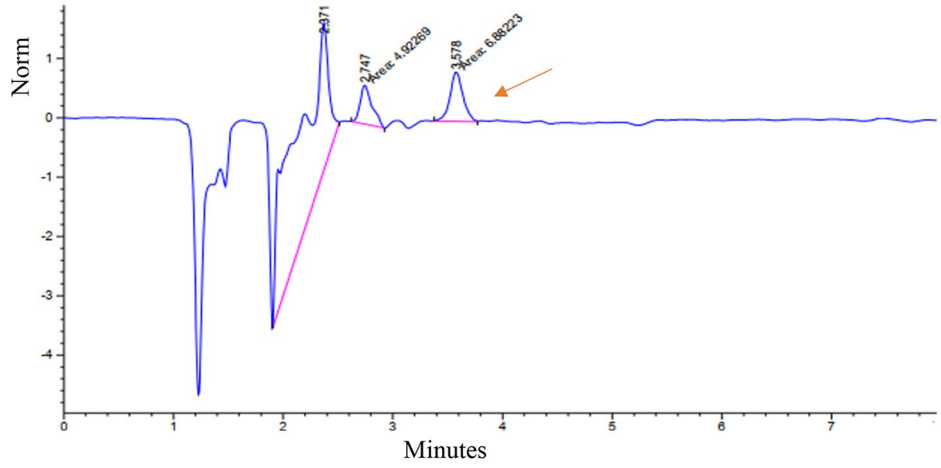
respectively, which was higher than the finding reported in other parts of Ethiopia.[18] The variation in the levels of oxytetracycline residues in the tissue samples could be due to exposure of the animals to antibiotics weeks or even days before slaughter, unauthorized use of the antimicrobials, over use of the drug, inadequate knowledge of the farmers and/or failure to apply instructions on the drug label.[22]

To determine the health risks of exposure to oxytetracycline, the results of the current study were compared with the maximum residue limits established by the FAO/WHO in 2010. According to WHO/FAO,[34] the MRL in muscles and kidney is 200 and 1200 µg/kg, respectively. Based on these recommended levels, the current study found that about 22.0% of samples from Dire Dawa and 17.8% from Harar slaughterhouses had concentrations of oxytetracycline higher than the maximum residue limits (MRLs).[34] This is relatively lower than the results reported in Addis Ababa and Nazareth slaughterhouses, which reported 48.3% and 48.1%, respectively.[18] Similarly, it was lower than the finding of another study conducted in South Africa, which reported about 63% of the analyzed samples contained chlortetracycline and oxytetracycline residues at levels that are lower than standard.[39] The variation in oxytetracycline residues may be due to the difference in exposure of the animals to antibiotics or variation in withdrawal time before slaughtering.

However, it was in line with the finding of the study conducted in Saudi Arabia which reported a concentration accounted for 15% of the samples that exceeded the MRL.[7] The study conducted in India reported a higher prevalence, which accounted for 33%.[37] The variation may be related to the types of samples analyzed to determine antibiotics, the latter being used in seafood. However, the study conducted in Tanzania reported that none of the samples contaminated with oxytetracycline exceeded MPL.[38] In the current study, none of the kidney samples had oxytetracycline exceeded MRL that was consistent with the finding reported in other parts of Ethiopia, which reported only one kidney sample exceeded MRL.[18]

Overall, these findings suggest that approximately about 22.0% of muscle samples collected from Dire Dawa and 17.8% from Harar town had oxytetracycline residues above maximum residual limits, which could have potential health implications for consumers. The high prevalence of oxytetracycline residues observed in the current study probably reflects cattle have sold for slaughter whilst under a therapeutic or prophylactic regimen or animals being slaughtered before the end of the withdrawal period.[40]

In addition, this may be due to the unreasonable use of large quantities of drugs without a professional prescription, the relatively cheap intake of antibiotics, and the inappropriate intake of antibiotics. Again, this antibiotic is widely used in these areas of research, probably due to their affordability, accessibility, and broad-spectrum effect. In addition, this may be due to lack of awareness and outreach, which may lead to drug abuse and overuse, and may result in failure to observe discontinuation periods.[41,42] This indicates that there is a need to take appropriate action by the concerned bodies to protect the consumer's health. The current study also recommends further research, particularly to assess the factors that increase the risk of antibiotics.



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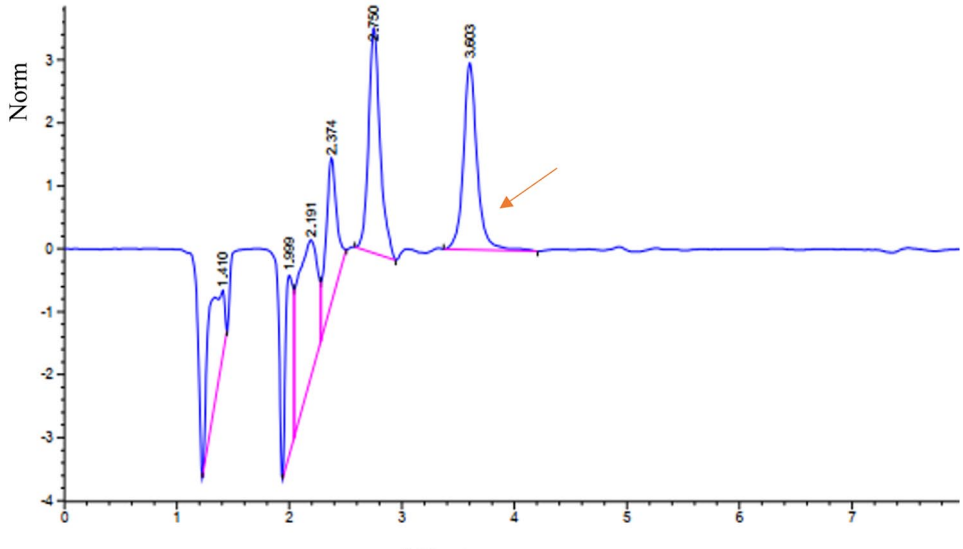

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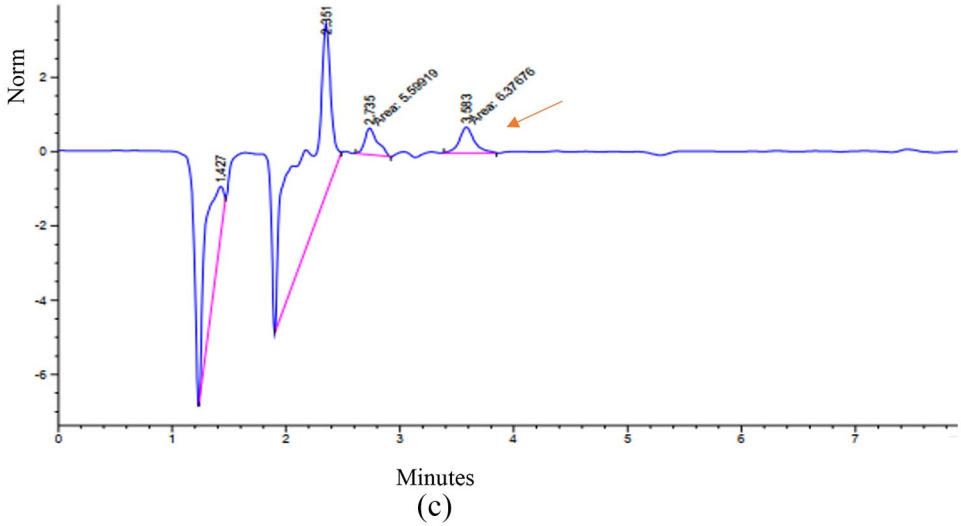

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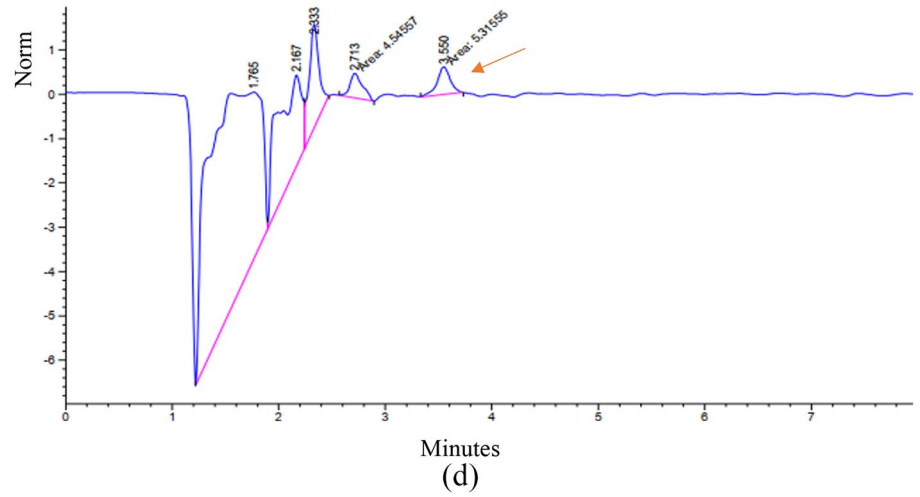
Figure 7. (Continued)



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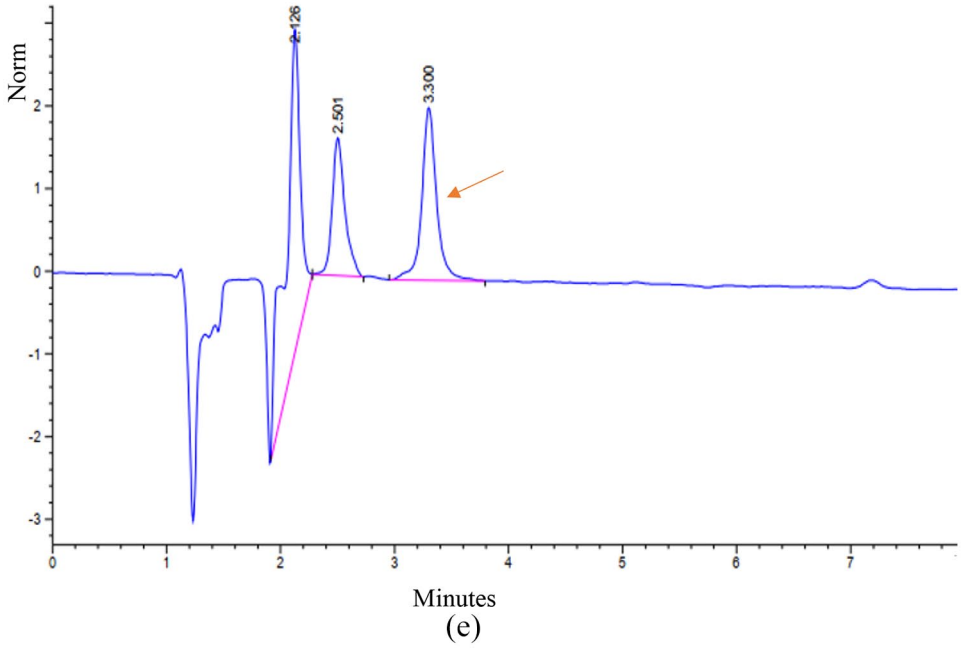

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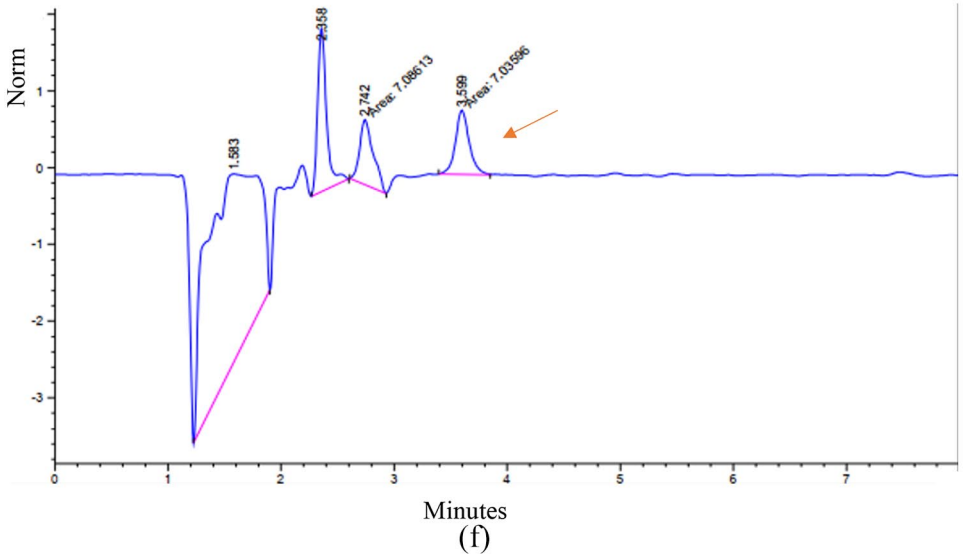

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Figure 7. (Continued)

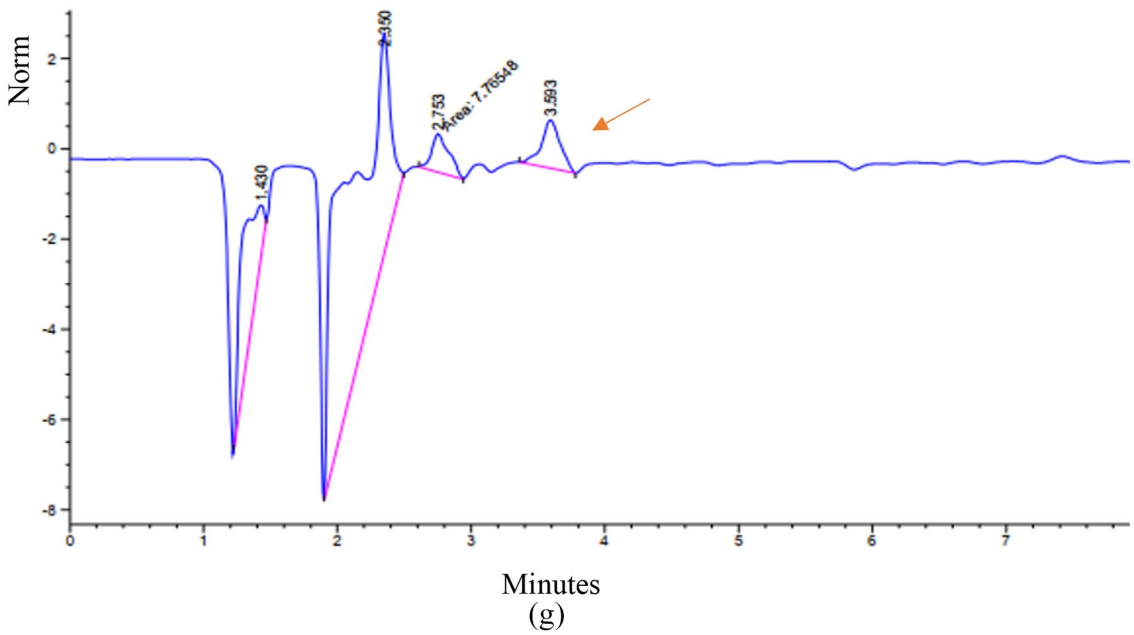

Figure 7. Some results of oxytetracycline residue in analyzed samples.

Conclusions

The current study revealed that about 22.0% of muscle samples collected from Dire Dawa and 17.8% from Harar town had oxytetracycline residues above maximum residue limits, which may have a potential health impact on the consumers. However, no kidney samples had a concentration of oxytetracycline higher than the recommended level. The high prevalence of oxytetracycline residues might be due to the irrational utilization of large amounts of drugs, without prescription by professionals, comparatively cheaper antibiotic intake, and inappropriate doses of antibiotics. It may be as a result of inadequate awareness, and insufficient extension activities that can lead to misuse and overuse of the drug and possibly failure to observe withdrawal periods. In order to protect the consumers' health, the concerned organizations, including Ethiopian Veterinary Drug and Animal Feed Administration and Control Authority, Food, Medicine and Healthcare Administration and Control Authority and Ministry of Health must take an appropriate measures. The obtained results are reliable in providing information on the quantities and health risks of oxytetracycline residues in beef meat. Furthermore, the authors recommend that there is a need for further comprehensive research that covers a wide location or geographical areas, including eastern Ethiopian towns. This finding indicates a need for a continuous improvement in farming practices in order to improve food safety.

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Author Contributions

NM conceived the idea and collected the data, and played a major role. The authors (DAM, AA, DB, and SM) contributed to data analysis, writing, and editing the document. NM, DAM, AA, DB, and SM gave valuable ideas for the manuscript and revised the manuscript. Finally, the authors read and approved the final version to be published and agreed on all aspects of this work.

Data Availability Statement

Almost all data are included in this study. However, additional data will be available from the corresponding author upon reasonable request.

Ethical Consideration

Ethical approval for this study was obtained from College of Veterinary Medicine, Haramaya University. Informed consent was obtained from all individuals included in this study.

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