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Depletion Time of Chlortetracycline in Broiler Chicken after Oral Administration and Health Risk Assessment

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Authors' Contribution
MIC designed the study, conducted experiments and drafted the manuscript. JJS conceived the idea, provided resources and reviewed manuscript. UM analyzed data and interpreted results. MMS facilitated in dose administration, sampling and care of experimental animals. MY contributed in health risk assessment and made illustrations.

Key words
Food safety, Pharmacokinetics, Edible tissues, Offals, Immunosorbent assay, Risk assessment

ABSTRACT

The depletion and distribution of chlortetracycline (CTC) was studied in broiler chicken. Total 21 poultry birds (average weight 2 Kg) were divided into treated (n=18) and control (n=3) groups. A treated group was given dose of CTC orally at 20 mg/Kg body weight continuously for 3 days. Treated chicken were slaughtered after each 24 h (up to 168 h) for the collection of selected edible tissues and offals (including liver, kidney, heart, lungs, gizzard, intestine, thigh and chest tissue) which were preserved at -20°C for further analysis. For CTC residues analysis, commercial ELISA kits (FOOD-1082-01D, MaxSignal) were standardized by calculating inhibition concentrations as 0.3 ppb (IC_{20}) and 2.0 ppb (IC_{50}). Validation studies were performed by spiking known negative (blank) samples at above and below the EU maximum residue level (MRL) *i.e.* 100 ppb and recoveries were calculated from 76.2 to 99.1%. Overall, results indicated that the CTC residues gradually decreased up to 5th day (120 h) but having residues concentration above the MRL in almost all tissue matrices while later on it further reduces below 100 ppb in next samplings of 144 and 168 h. Surveillance studies were also conducted to evaluate CTC dietary risk assessment through market samples of poultry meat. Out of 80 samples, health risk index (cut off vale one) exceeded in 7 samples indicated 8.75% possibility of high health risk linked with consumption of CTC residues contaminated broiler meat. This information may be helpful to the policy makers, health specialists, producers, consumers and all other stakeholders involved in poultry production.

INTRODUCTION

Tetracyclines (TET) are commonly used antimicrobials in poultry industry for prophylactic purposes and as growth promoters to increase production by improving feed efficacy. Approximately 90% antimicrobials are used at sub-therapeutic concentrations, out of which 70% are used to control diseases while 30% are used for growth promotion (Jayalakshmi *et al*., 2017). Due to broad spectrum activity, TET are frequently used to treat many diseases like salmonellosis, staphylococcal infections, etc. The effectiveness of the antimicrobials is mainly depending upon route of administration which ultimately linked with residues distribution among tissues (Reig and Toldra, 2008).

In developing countries like Pakistan, chlortetracycline (CTC) and oxytetracycline (OTC) are extensively used in poultry production which may leave residues in edible tissues and animal products. So, it is important to monitor and control antimicrobial residues in those animals which are slaughtered without following withdrawal period. There are many factors for accumulation of CTC residues in poultry tissues including overdosing, misuse and insufficient withdrawal time. Such residues may cause health hazards issues in consumers like microbiological, toxicological and immunological disorders. The antimicrobial residues in poultry products may cause allergic reactions. Risk assessment brings together hazard characterization and exposure assessment (Omeiza *et al*., 2012).

Antimicrobial residues in animal-derived food are potential threat to public health. So, it is necessary to control residue in animal-derived food (Sanli *et al*.,

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2023). There is another potential hazard is development of resistance in commensal strains of medicated animals which becomes a problem as the resistant genes can be transferred to human (Peeters *et al*., 2018) and they will not respond against treatments during illnesses (Marchetti *et al*., 2012). Mund *et al*. (2017) indicated direct relation of antimicrobials to poultry production and their residues have been reported in meat and eggs (Muaz *et al*., 2018). However, for public health safety, European Union has established maximum residue limits (MRLs) through Commission Decision 37/2010/CE to reduce the potential exposure to antimicrobials residues. The EU MRL for CTC in all food-producing species is 100 µg/Kg, 300 µg/Kg and 600 µg/Kg for muscle, liver and kidney, respectively.

TET are poorly absorbed by animal tissues and their major part is excreted out in unaltered form (Arikan *et al*., 2009). Many researchers reported the accumulation and detection of TET residues up to 20 mg L^{-1} in fertilized soils (Daghrir and Drogui, 2013). The rate of drug residues depletion from tissues depends upon dose, method of administration, animal species and formulation of a given drug. Withdrawal period may also be influenced by quantity and quality of drinking water by animals. Depletion profile studies are essential to recommend appropriate withdrawal time (Mestorino *et al*., 2018). Earlier, pharmacokinetics behaviour of OTC was studied in edible tissues of broiler chicken by giving single dose of OXTRA L.A formulation via intramuscular route. The broiler breast muscle exhibited OTC lower than MRL after 120 h while other edible tissues needed more withdrawal time (Mumtaz *et al*., 2021).

Keeping in view the importance of withdrawal period of antimicrobials, present studies were performed to observe the distribution and depletion of widely used CTC in broiler chicken. Similarly, surveillance studies were also conducted to monitor CTC residues in broiler meat samples, collected from local markets of District Faisalabad (Punjab), Pakistan. The generated data was further used for health risk assessment.

MATERIALS AND METHODS

Apparatus and chemicals used

Chlortetracycline ELISA kits (Cat. #. FOOD-1082-01D, MaxSignal), n-Hexane (VWR), ELISA reader (ELx808, BioTek), ELISA washer (ELx50, BioTek), Refrigerated centrifuge (5340R, Eppendorf), Homogenizer (HG-15D, DAIHAN Scientific), Vortexer (Lab-Line), TurboVap® system (Biotage), Falcon tubes (50 mL capacity, VWR), Glass test tubes (Kimax), ELISA plate sealers.

Experimental design

Twenty-one broiler chickens (average weight 2 Kg) were obtained from control shed with known history of using antimicrobials and maintained in Animal House, NIAB, Faisalabad (Pakistan). These broiler chickens were arranged in to treated (n=18) and control (n=3) group. Commercially available TETRACHLOR® powder was dissolved in double distilled water and given orally @20 mg/ Kg body weight to each treated broiler chicken continuously for 3 days. Samples were collected as per VICH Guidelines (GL 48), for which 3 treated broiler chickens were slaughtered every 24 h and different offals and edible tissue samples including liver, kidney, heart, lungs, gizzard, intestine, thigh muscles and chest muscles were collected and stored at -20 °C for further analysis (Fig. 1).

Determination of CTC residues in tissues

Samples were extracted by following commercial kit manual (FOOD-1082-01D, MaxSignal). Tissue samples were cut down in to small pieces and homogenized at 10,000 rpm for 1 min. Homogenized samples (1 g) were taken in 15 mL plastic tubes then added 3 mL of 1X extraction buffer (5X concentrated extraction buffer provided with kit) and 1 mL n-hexane. After vortexing for 15 min in multi-tube vortexer, samples were centrifuged at 4000 × *g* for 10 min. The upper n-hexane layer was discarded and 200 µL supernatant in glass tubes that already contained 300 µL of 1X sample diluent (10X concentrated sample diluent provided with ELISA kit). After vortexing for 1 min, 75 µL of extract was used for assay development.

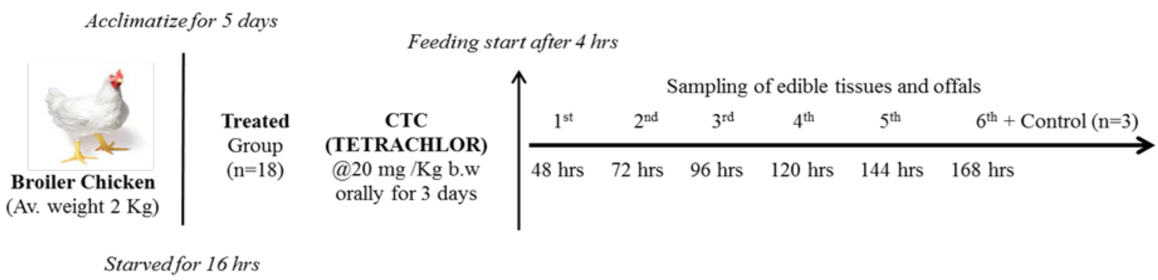

Fig. 1. Experimental design to study withdrawal time of CTC in broiler chicken.

For assay performance as per plate layout, 75 µL of both standards and samples were added on plate in triplicate. Then 100 µL of antibody solution was added in all used wells. After mixing solution for 1 min, plate was covered with plate sealer and incubated for 55 min at room temperature. The plate was washed for 3 times with 1X wash solution. After washing, 100 µL of TMB substrate was added in each well and placed in incubator for 15 min in dark at room temperature. After incubation, 100 µL of stop solution was added in each well to stop the reaction and measured optical density at 450 nm. The relative absorbance (RA) was calculated from optical density to construct calibration curve which was further used to calculate inhibition concentrations (IC_{20} and IC_{50}). For validation studies, recoveries (%) were calculated by spiking known negative tissues (chest muscle, thigh muscles, heart, kidney and liver) with above and below the MRL 100 ng/mL (50, 100 and 200 ppb standards). The concentration of unknown samples was determined by interpolating RA of unknown samples in calibration curve.

$$\text{Relative Absorbance} = \frac{\text{Absorbance of standard (or sample)}}{\text{Absorbance of zero stnadard}} \times 100 = \frac{B}{B_0}\ (\%)$$

Surveillance studies for health risk assessment

During 2022-23, total 80 meat samples of broiler chicken were collected from local markets at 25 different locations of District Faisalabad, Pakistan by covering area of 30 km radius from city centre. These samples were packed in labelled zip bags and transferred in chilled condition (4-6ºC) within 6 hrs to Food Safety Labs (ISO-17025 accredited) at Nuclear Institute for Agriculture and Biology, Faisalabad which were analysed for CTC residues.

Health risk for CTC residues was estimated on the basis of chicken meat instead of offals as the most of population is chicken meat consumer. Average consumption of broiler meat in Pakistan was 22.5 g/day/capita as reported by Pakistan Economic Survey (2020-21). The maximum residue limit of CTC (100 ppb) was considered as its acceptable daily intake. The estimated daily intake (EDI) was calculated by following formula reported by Chughtai *et al*. (2021). Similarly, the health risk index (HRI) was estimated by using formula given by Hamid *et al*. (2017).

$$\text{EDI} = \frac{\text{Meat Residual CTC conc.} \times \text{Meat consumption (Kg/day)}}{\text{Body weight (60 Kg) foran adult}}$$

$$\text{HRI} = \frac{\text{Estimated CTC daily intake)}}{\text{Acceptable CTC daily intake}}$$

RESULTS

Standardization and validation of immunosorbent assay (ELISA)

The ELISA kits were standardized by using concentrations of 0, 0.15, 0.4, 0.8, 1.6, 9.38 and 18.75 ppb. From calibration curve, the IC_{20} and IC_{50} were calculated as 0.3 ppb and 2.0 ppb, respectively. The R.A (%) was found inversely proportional to the CTC concentration. A linear regression was obtained (y = -15.73ln(x)+60.758) with R^2 value 0.9825 as shown by Figure 2. Final concentration was determined by multiplying with dilution factor 10. Kits showed 100% cross reactivity with CTC and have limit of detection 0.5 ppb.

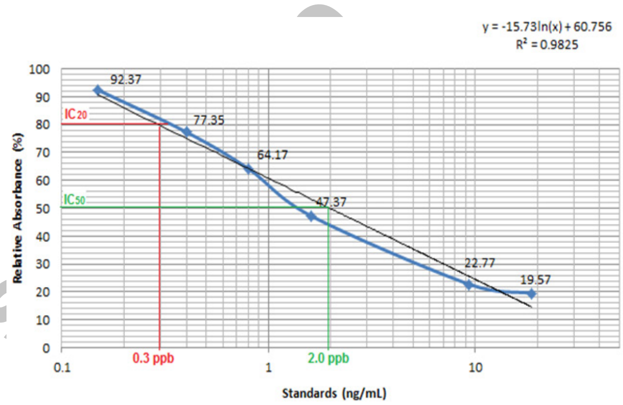

Fig. 2. Calibration curve for determination of CTC residues in different tissues of broiler chicken.

Table I. Recovery (%) of CTC residues in selected tissues of broiler chicken at different spiking levels.

Spiking level	Tissue matrix	Mean O.D	R.A (%)	Final conc. (ppb)	Recov-ery (%)
½ MRL (50 ppb)	Chest muscle	1.279	47.40	45.62	91.24
	Thigh muscle	1.299	48.12	43.56	87.12
	Heart	1.295	47.96	44.02	88.04
	Kidney	1.267	46.92	47.03	94.06
	Liver	1.339	49.59	39.69	79.38
1 MRL (100 ppb)	Chest muscle	1.062	39.33	76.19	76.19
	Thigh muscle	0.977	36.20	92.97	92.97
	Heart	1.012	37.47	85.78	85.78
	Kidney	1.041	38.55	80.06	80.06
	Liver	0.987	36.55	90.91	90.91
2 MRL (200 ppb)	Chest muscle	0.656	24.29	198.20	99.10
	Thigh muscle	0.716	26.51	172.09	86.04
	Heart	0.706	26.15	176.16	88.08
	Kidney	0.689	25.51	183.38	91.69
	Liver	0.727	26.92	167.65	83.82

OD: Optical density; RA.: Relative absorbance.

As per guidelines of VICH GL 49(R), the accuracy of test method was determined by calculating recovery (%) which was found in acceptable range. At 50, 100 and 200 ppb spiking levels, the recovery was ranged from 79.4 to 95.1%, 76.2 to 92.9% and 83.8 to 99.1%, respectively (Table I). Similarly; the method precision as coefficient of variation (CV) within-run (intra-assay) was found 6.29, 8.32 and 6.66% and between-run (inter-assay) was 11.7, 8.95 and 9.32% at 50, 100 and 200 ppb spiking levels, respectively.

Distribution and depletion of CTC residues in offals and edible tissues

Distribution studies indicated that maximum CTC depletion (95.04%) was found in heart (from 331.2 to 16.4 ppb) while minimum (81.29%) in kidney (from 432.6 to 80.9 ppb) in 168 h. Depletion (%) gradually increased in all tissues up to 7th day of sampling from 25.3% to 55.3% (Table II).

Overall, results indicated that the CTC residues gradually decreased up to 5th day (120 h) but concentration remained above the MRL in almost all tissue matrices while later on it further reduces below the MRL in 6th day (144 h) and 7th day (168 h) samples as shown in Figure 3.

Prevalence of CTC residues and health risk assessment

The CTC residues were determined in 80 meat samples of broiler chicken, collected from different locations of District Faisalabad. Overall results indicated that CTC residues were detected in 24% meat samples. Out of 19 samples containing CTC residues, 7 samples (8.75%)

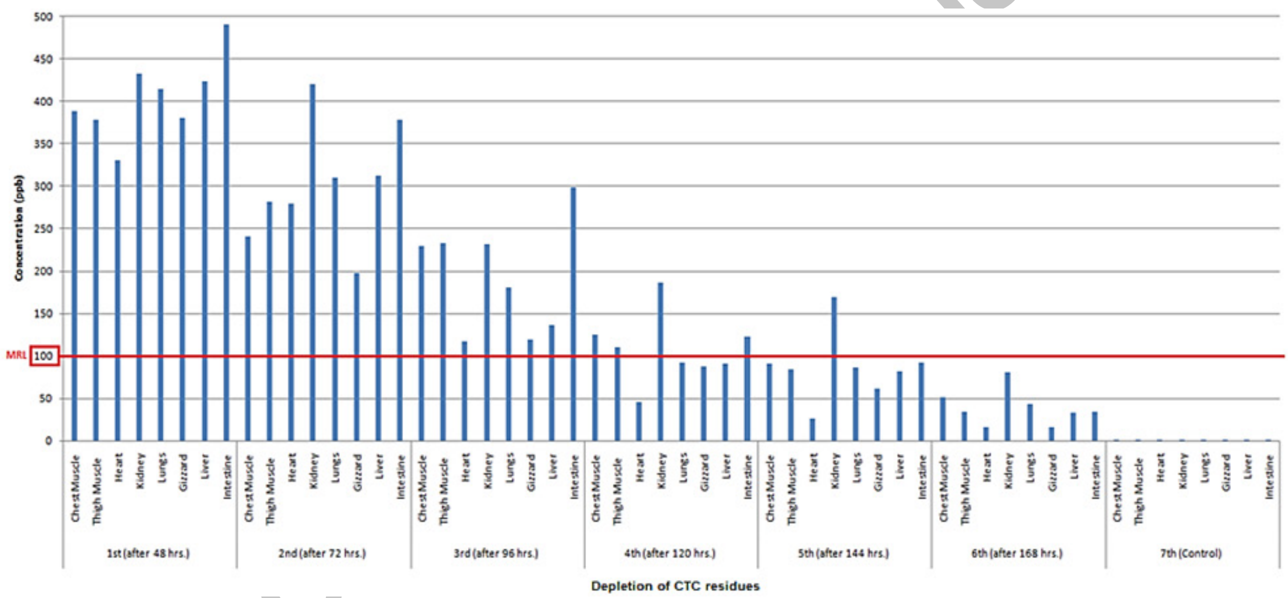

Fig. 3. Depletion trend of chlortetracycline residues in different tissues of broiler chicken up to168 h.

Table II. Mass balance of CTC (administered dose 20 mg/Kg) in broiler chicken over a period of 7 days.

Parameters	Concentration of Chloramphenicol (ppb) in treated samples						Overall depletion (%)
	Day 2 (48 h)	Day 3 (72 h)	Day 4 (96 h)	Day 5 (120 h)	Day 6 (144 h)	Day 7 (168 h)	
Liver	423.5±22.5	312.2±19.8	136.2±13.5	91.1±7.33	82.3±5.23	32.8±3.14	92.26
Heart	331.2±17.8	279.5±21.4	117.2±11.7	45.8±5.13	26.3±3.44	16.4±2.10	95.04
Intestine	491.3±27.8	378.3±17.5	298.6±15.6	122.9±10.1	92.4±7.21	34.3±3.38	93.03
Chest muscle	388.7±20.1	240.4±12.7	229.6±12.8	125.2±8.45	90.8±6.78	51.8±4.40	86.67
Thigh muscle	378.7±21.4	281.5±11.2	233.4±16.2	110.2±9.45	84.8±5.41	34.3±4.12	90.95
Kidney	432.6±23.8	420.6±27.1	231.5±11.8	186.2±12.5	169.5±10.7	80.9±4.66	81.29
Lungs	415.1±31.8	309.7±21.2	180.8±10.2	91.9±7.94	86.7±8.11	43.9±3.41	89.42
Gizzard	380.9±23.7	198.2±14.5	119.1±9.45	87.2±6.32	61.6±4.57	19.4±2.11	94.91
Total Conc.	2750.76	2042.06	1247.85	737.67	601.97	276.43	90.42
Depletion (%)	-	25.34	36.12	44.35	19.31	55.26	-

Values are mean of three replicates ± S.D

Table III. Health risk assessment in relation to consumption of CTC contaminated broiler meat.

Sample code	Conc. (ppb)	Daily intake (ppb)	HRI	Health risk	Sample code	Conc. (ppb)	Daily intake (ppb)	HRI	Health risk
CTC in Broiler Meat (High risk = 8.75%, Low risk = 16.25%, Safe = 75%)									
BMT-22-002	24.6	0.0092	0.246	Low	BMT-22-045	29.9	0.0112	0.299	Low
BMT-22-005	114	0.0428	1.140	High	BMT-23-004	13.9	0.0052	0.139	Low
BMT-22-010	38.5	0.0143	0.385	Low	BMT-23-009	153	0.0574	1.530	High
BMT-22-015	85	0.0319	0.850	Low	BMT-23-013	11.6	0.0043	0.116	Low
BMT-22-020	13.7	0.0052	0.137	Low	BMT-23-019	118	0.0442	1.180	High
BMT-22-024	115	0.0431	1.150	High	BMT-23-023	101	0.0378	1.010	Low
BMT-22-025	108	0.0405	1.080	High	BMT-23-027	66.4	0.0249	0.664	Low
BMT-22-030	35.6	0.0131	0.356	Low	BMT-23-030	25.5	0.0090	0.255	Low
BMT-22-034	14.9	0.0056	0.149	Low	BMT-23-033	141	0.0528	1.410	High
BMT-22-040	118	0.0443	1.180	High	BMT-23-035	11.1	0.0043	0.111	Low

BMT: broiler meat; HRI: health risk index; Cut-off value for HRI = 1 set at MRL ≤100 ppb.

were found positive with maximum residues 153 ppb. The health risk linked with CTC residues in broiler meat was measured by calculating HRI. It's cut off value was set at 1 which was equivalent to the MRL of CTC residues. The HRI value more than one indicated high risk, values between zero to one considered as low risk and without CTC residues were safe for health. Out of 80 samples, HRI exceeded in 7 samples indicated possibility of high health risk linked with consumption of CTC residues contaminated broiler meat. Detection rate of CTC was calculated 24% in broiler meat samples with 8.75% high risk, 16.25% low risk and 75% safe (Table III).

DISCUSSION

Antimicrobials are widely used with overdoses in poultry to control diseases, promote growth and to enhance feed efficiency (Selaledi *et al*., 2020). Approximately 63151 tons antibiotics are being used in livestock production throughout the world, reported by Van *et al*. (2020). Sarker *et al*. (2020) reported indiscriminate use of antimicrobials in livestock sector for therapeutic and growth promoting purposes. As a result, residues contaminated dairy products are being produced which pose serious impact on consumer's health. Similarly, the persistence of antimicrobial residue in humans may pose immuno-pathological effects which may cause bone marrow toxicity (Palmieri *et al*., 2014). So, the safe and appropriate use of antimicrobials is one of the most challenging public health issues during last decade.

The incorrect use of antimicrobials in chickens may leave residues in eggs and tissues at concentrations above the MRL that may be harmful to human health (Goetting *et al*., 2011; Kehinde *et al*., 2012). While consuming less contaminated food with low residue level, will not pose serious health effects in consumers (Vishnuraj *et al*., 2016). Due to continuous use of antimicrobials in poultry to control diseases and mortality, the contaminated food is produced and marketed which is of significant public health concern (Chota *et al*., 2021).

Sattar *et al*. (2014) also reported 20% deposition of TET residues in thigh muscles and 24% in breast muscles of broilers. Karmi (2014) also confirmed the presence of TET residues in market chicken meat samples (fresh, local frozen and imported frozen breast and thighs) sold for human consumption. Bahmani *et al*. (2020) performed TET monitoring studies in meat (chicken, turkey, cow, quail, calf, goat, sheep, trout and shrimp) and milk (cow, sheep and goat) samples collected from Tehran and analysed by using ELISA and HPLC-UV. Maximum TET concentration was observed 155.4 µg/Kg in chicken meat and in cow milk 104.3 µg/L. Anadon *et al*. (2012) reported mean concentration of CTC in kidney, liver and muscles as 835.3, 192.7 and 126.3 µg/Kg, respectively, after 24 h of final oral dose administration in broiler chicken. The CTC residues were detected in kidney (205.4 µg/Kg) and liver (81.7 µg/Kg), but not in muscle, 3 days after the end of CTC treatment.

Sanli *et al*. (2023) analysed bovine kidney, chicken gizzard and eggs (collected from Turkish market) for TET residues by using RP-LC method. Results indicated the presence of OTC, TET and CTC residues in 14 (30.43%) kidney samples. CTC was detected in 7 (16.67%) chicken gizzard samples. Similarly, the analysis of eggs also indicated the presence of TET residues in nine samples (8.82%) but only one (0.98%) sample has OTC concentration above the MRL (267.1 mg/Kg). Salama *et al*. (2011) found TET residues above MRL in breast (8%), thigh (8.67%) and liver (13.3%) samples.

Islam *et al*. (2022) analysed 112 homemade and commercial poultry feed samples (collected from different districts of Bangladesh) for OTC and CTC residue by using thin-layer chromatography. Results indicated all homemade feed samples (100%) were found positive for both OTC and CTC residues while (95%) of commercial feed tested positive for antibiotic residue in all four districts. The occurrence of OTC in soil has been extensively reported in crop fields and animal production farms (Zhou *et al*., 2013). Cornejo *et al*. (2018) studied depletion of CTC in broiler chickens, treated with 20% CTC. Results indicated that the presence of CTC residues in droppings from 179.5 to 665.8 g/Kg. On the basis of this data, they set depletion time as 69 days for chicken droppings.

Kodimalar *et al*. (2014) assessed 12-layer farms for quantification and confirmation of CTC residue in eggs by using HP-TLC. Results indicated that CTC residues in eggs increased up to the 7th day at all farms and then started to decrease on 9th day and further quickly decreased on 14th day. The mean CTC concentration in eggs was found 206 µg/Kg on 7th day. Abdel-Mohsein *et al*. (2015) conducted study to analyse TET residues in 282 poultry tissues samples collected from poultry farms at Upper Egypt. The results indicated the presence of OTC residue in 50% muscles, 100% liver and 93% kidney samples. While TC residues were found in 69% muscles, 72% liver and 86% kidney samples. The highest concentration of both OTC and TET was observed in liver as 7.23 µg/g and 1.06 µg/g, respectively.

CONCLUSION

Chlortetracycline (CTC) showed depletion trend as residues gradually decreased up to 120 hrs (5th day) but concentration remained above the MRL (100 µg/Kg) in almost all tissue matrices while later on it further reduces below the MRL in next samplings. So, withdrawal time may be recommended or set at 7 days as all samples were found in safe limit after 158 hrs. Surveillance data indicated the presence of CTC residues in 7 commercial broiler meat samples (out of 80) with concentration exceeding MRL. Health risk indices highlighted the possibility of 8.75% potential health risk in humans associated with consumption of CTC contaminated broiler meat. Best management practices and continuous monitoring may be helpful to avoid or reduces the chances of contamination at poultry farms.

DECLARATIONS

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IRB approval

This study and all the experimental procedures were approved by the Annual In-house Review Committee of NIAB, Faisalabad.

Ethical statement

Animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Sciences Division, NIAB Faisalabad (Approval No. NIAB/2023-003). All procedures were conducted in accordance with national and international guidelines for the ethical care and use of laboratory animals.

Generative AI and AI-assisted technology statement

The authors declare that no generative artificial intelligence (AI) tools or AI assisted technologies were used in the preparation, writing, data analysis or editing of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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