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Ampicillin Pharmacokinetics in Broiler Chickens and its Implications on Public Health

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Authors' Contribution
MIC designed the study, conducted experiments and drafted the manuscript. JJS conceived the idea, provided all 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
Depletion, Ampicillin, Edible tissues, Offals, ELISA, Risk assessment

ABSTRACT

The pharmacokinetics of ampicillin (AMP) was investigated in broiler chickens to assess residue depletion and potential public health risks. A total of 24 birds (average weight: 1.5 kg) were divided into treated (n=21) and control (n=3) groups. The treated group received AMP (AMPICOX®) at a dosage of 40 mg/kg body weight via intramuscular injection for three consecutive days, while the control group remained untreated. Samples from edible tissues and selected offal (including the liver, kidney, heart, lungs, intestine, thigh, and breast muscles) were collected every 24 h (up to 168 h) and stored at -20°C for analysis. Residue detection was performed using commercial ELISA kits (E4350-100, BioVision®), with inhibition concentrations standardized at 17.5 ppb (IC_{20}) and 112 ppb (IC_{50}). Validation studies were conducted by spiking blank tissue samples at levels above and below the maximum residue limit (MRL) of 50 µg/kg, showed recoveries ranging from 78.7 to 100%. The results indicated that AMP residues gradually declined over 96 h; however, residue concentrations remained above the MRL in most tissues. In next samplings, it further reduces below MRL after 120, 144, and 168 h of post-administration. Additionally, a surveillance study was conducted to assess potential health risks. Among 65 analysed samples, six (9.23%) exceeded the health risk index cut-off value of one, suggesting a potential public health concern associated with the consumption of AMP-contaminated broiler meat. These findings provide valuable insights for policymakers, health professionals, poultry producers and consumers regarding antibiotic residue management in poultry production.

INTRODUCTION

Ampicillin (AMP) is a widely used broad-spectrum antibiotic from the aminopenicillin group. It is cost-effective, easily accessible and characterized by low toxicity, high water stability and resistant to gastric acid (Gawronska *et al*., 2022). It is commonly incorporated into animal feed to treat and prevent various diseases including gastrointestinal, urinary, and respiratory infections (Wang *et al*., 2024). Additionally, it is effective in managing necrotic enteritis caused by *Clostridium perfringens* (Youssif *et al*., 2023) and to control secondary infections associated with chronic illnesses caused by *E. coli*, *Pasteurella multocida* and *Salmonella* spp. in poultry (Chen *et al*., 2019). AMP exerts bactericidal effects by inhibiting transpeptidase activity, disrupting glycopeptide synthesis, and preventing bacterial cell wall formation, leading to cell death (Karunarathna *et al*., 2024).

The bioavailability and pharmacokinetic properties of antibiotics are essential in food-producing animals, as they help to maintain effective drug concentrations and influence residue levels in various food products. Ensuring the efficacy and safety of antibiotics in poultry, is a key focus in veterinary pharmacology and toxicology with significant implications for food safety and animal health (Chen *et al*., 2019). Additionally, prolonged and improper antibiotic use can contribute to bacterial resistance, diminishing antimicrobial effectiveness (JECFA, 2012).

Extensive research has been conducted on the pharmacokinetics of β-lactam antibiotics, particularly

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aminopenicillins in animal systems. Studies have explored the oral administration of AMP (Jerzsele *et al*., 2009) and its intramuscular use as well as amoxicillin (AMOX) in broiler chickens (El-Sayed *et al*., 2014). Additionally, research has examined AMP in combination with sulbactam in goats (Espuny *et al*., 1996), AMOX with clavulanic acid in domestic hens (Shannon *et al*., 2020), AMP sodium and AMP trihydrate in llamas (Kreil *et al*., 2014) and AMP alongside its prodrug bacampicillin (BAC) in chickens and turkeys (Guzelaydin *et al*., 2025).

However, administering higher sub-therapeutic doses of β-lactam antibiotics in food-producing animals can lead to unwanted residues in milk and tissues, posing a potential risk to individuals with penicillin hypersensitivity (Moga *et al*., 2021). Frequent use of antibiotics can lead to various adverse effects on the reproductive system, including an increase in sperm abnormalities (Mohammedi *et al*., 2024). AMP has also been associated with histopathological changes in the reproductive organs, liver, and kidneys (El-Sawy *et al*., 2018). Overuse of penicillin-based drugs may also disrupt the balance of human gastrointestinal microbiota, potentially leading to health risks.

Studies have shown that most lactic acid bacteria found in fermented milk exhibit resistance to penicillin, with only 10% being susceptible to AMP (Lavanya *et al*., 2011). Similarly, research in Malaysia revealed that many lactic acid bacteria species isolated from broiler chicken feces were resistant to AMP due to prolonged exposure to the antibiotic as a growth promoter and therapeutic agent (Shazali *et al*., 2014). Therefore, it is crucial to educate farmers on the safe and responsible use of antibiotics in animal farming to mitigate the risks associated with antimicrobial resistance (Hall *et al*., 2011).

Health concerns including antimicrobial resistance are linked to the excessive consumption of products suspected to contain antibiotic residues. Antimicrobial resistance is a growing global threat to public health, undermining the progress achieved through antibiotic discovery. Bacterial resistance genes from the animal microbiome can be transferred to human microbiota, further exacerbating this issue (Oyedeji *et al*., 2019). Regulatory authorities and veterinarians must enforce drug withdrawal periods before slaughter. Regular monitoring of drug residues in poultry tissues is essential for consumer safety (Mund *et al*., 2017).

The World Health Organization (WHO) has highlighted the urgent threat of antimicrobial resistance, cautioning that without immediate action to regulate antibiotic use in both human and veterinary medicine, their effectiveness could be severely compromised. To safeguard consumer food safety, the European Union (EU) has prohibited the use of antibiotics as feed additives and established a maximum residue limit (MRL) for AMP at 50 µg/kg in all food producing species (muscle, kidney, liver and fat) and 4 µg/kg in milk (EU Regulation No. 37/2010).

Considering the importance of bioavailability and pharmacokinetics of antibiotics in animal systems, this study was designed to examine the distribution and depletion of AMP in broiler chickens. Additionally, surveillance studies were conducted to detect AMP residues in broiler meat sold in District Faisalabad (Punjab), Pakistan. The collected data was further analysed to assess potential health risks associated with the consumption of residue-contaminated meat.

MATERIALS AND METHODS

Apparatus and chemicals used

ELISA reader (ELx808, BioTek), ELISA washer (ELx50, BioTek), Refrigerated centrifuge (5340R, Eppendorf), Homogenizer (HG-15D, DAIHAN Scientific), Vortexer (Lab-Line), TurboVap® system (Biotage), AMP ELISA kits (Cat. #. E4350-100, BioVision®), Falcon tubes (50 mL, VWR), Glass test tubes (Kimax), ELISA plate sealers.

Dosage administration in experimental broiler chickens

A total of 24 broiler chickens (average weight: 1.5 kg) were obtained from a controlled shed with a documented history of antimicrobial use and transferred to the Animal House at NIAB, Faisalabad (Pakistan) for distribution and depletion studies. The birds were divided into two groups: a treated group (n=21) and a control group (n=3). The commercially available AMP formulation (AMPICOX®) was used for treatment, with each 50 mL vial containing 6.25 g of AMP as trihydrate. The treated group received an intramuscular injection of 0.15 mL (equivalent to 40 mg/kg body weight) in the pectoral muscle once daily for three consecutive days. The control group remained untreated.

Collection of samples from treated and control chickens

Tissue samples were collected following VICH Guidelines (GL 48). In each sampling session, three treated broiler chickens were slaughtered, and various edible tissues and offal (including the liver, kidney, heart, lungs, intestine, thigh muscles, and chest muscles) were collected. Sampling was conducted at 24 h intervals (24, 48, 72, 96, 120, 144 and 168 h). Control birds were sampled alongside the treated group. All collected samples were stored at -20°C for further analysis. The experimental design is illustrated in Figure 1.

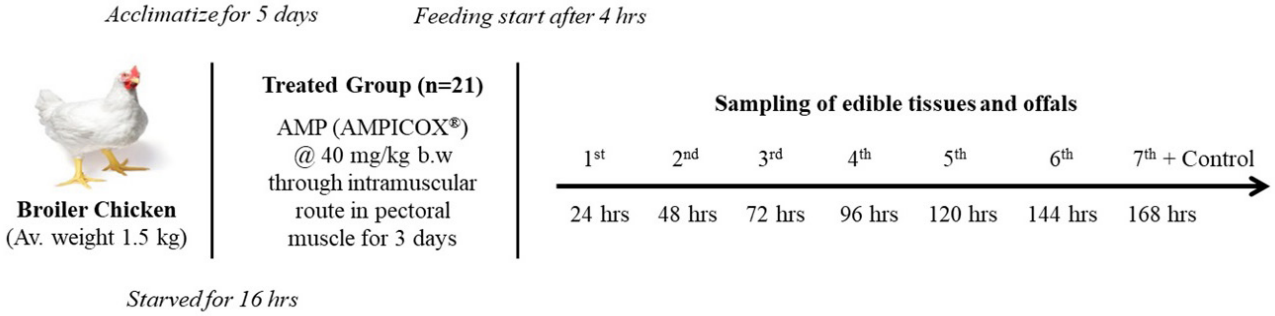

Fig. 1. Experimental design to study depletion of Ampicillin residues in broiler chicken.

Determination of AMP residues by immunosorbent assay (ELISA)

Extraction was performed according to the kit manual (E4350-100, BioVision®). Tissue samples were finely chopped and homogenized at 10,000 rpm for 1 min. Homogenized sample (1 ± 0.05 g) was transferred to a 15 mL plastic tube, mixed with 1 mL of double-distilled water and supplemented with 20 µL of extraction buffer. The mixture was vortexed for 5 min, followed by centrifugation at 10,000×g for 20 min at 4°C to separate the supernatant. The recovered supernatant was then diluted 10-fold using the sample diluent provided in the kit. After vortexing, 50 µL of the extract was used for assay development. Assay was performed by following the designated plate layout, 50 µL of both standards and samples were added to the plate in triplicate. Subsequently, 50 µL of enzyme conjugate was added to all wells. After mixing, 50 µL of antibody working solution was introduced and the plate was gently shaken to ensure proper mixing. The plate was then covered with a plate sealer and incubated for 60 min at room temperature. After incubation, the plate was washed five times with 1X wash buffer (250 µL per well). The plate was then blotted dry using absorbent paper. Next, 100 µL of TMB substrate was added to each well, and the plate was gently tapped to ensure thorough mixing. The plate was incubated in the dark at room temperature for 15 min. After incubation, 50 µL of stop solution was added to each well to terminate the reaction. Finally, optical density was measured at 450 nm.

Standardization and validation of ELISA kits

The commercial ELISA kits were standardized using a range of standards prepared from 625 ppb stock solution. Relative absorbance (%) was calculated from the optical density values to construct a calibration curve, which was subsequently used to determine the inhibition concentrations (IC_{20} and IC_{50}). For validation studies, matrix effect was assessed through recovery percentages by spiking known negative tissue samples (heart, liver, kidney, breast, and thigh muscles) with standards at concentrations above and below the EU MRL of 50 µg/kg *i.e.,* 25, 50, and 100 ppb (EU Regulation No. 37/2010). The mean optical density (OD) was used to calculate relative absorbance (RA) using the following formula:

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

The concentration of unknown samples was determined by interpolating their RA values on the calibration curve.

Surveillance studies for health risk evaluation

A total of 65 broiler chicken meat samples were collected in zip bags from local markets in District Faisalabad, Pakistan, during 2023-2024. The samples were transported under chilled conditions (4-6 ºC) to the Food Safety Laboratories (ISO/IEC 17025:2017 accredited) at the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad and stored at -20 ºC for further analysis. The health risk associated with AMP residues was estimated based on the assumption that the general population primarily consumes broiler chicken meat rather than offal. According to the Pakistan Economic Survey (2020–21), the average per capita broiler meat consumption is 22.5 g/day. The MRL for AMP was set at 50 ppb which was considered the acceptable daily intake (ADI). The estimated daily intake (EDI) was calculated using the formula provided by Chughtai *et al*. (2021). Similarly, the health risk index (HRI) was determined following the methodology described by Hamid *et al*. (2017).

$$\text{EDI} = \frac{\text{Residual AMP conc.} \times \text{Food consumption (Kg/day)}}{\text{Body weight for an adult (60 Kg)}}$$

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

Statistical analysis

Data were compiled and analyzed using Microsoft Excel 2019 (Microsoft Corporation, USA). Descriptive statistics, including means, standard deviations, coefficient

of variance (CV) and percentages, were calculated. Graphical representations were generated using Excel chart tools.

RESULTS

Standardization and validation of test method (ELISA)

The commercial ELISA kits were standardized using AMP concentrations of 0, 5, 25, 125, 312, and 625 ppb. Based on the calibration curve, the IC_{20} and IC_{50} values were determined as 17.5 ppb and 112 ppb, respectively. The relative absorbance (RA%) was found to be inversely proportional to the AMP concentration. A linear regression was obtained (y= -14.1ln(x)+115.7) with R^2 value 0.9755 as shown in Figure 2. The final concentration was determined by multiplying the obtained value by the dilution factor (10). The kits demonstrated 100% cross-reactivity with AMP and had a limit of detection (LOD) of 10 ppb.

The accuracy and precision of the test method was evaluated by following the VICH GL 49(R) guidelines. The accuracy was measured by calculating recovery percentage (%). In present study, the recoveries were calculated as 79.9-90.8%, 78.7-97.6% and 88.7-100% at spiking levels of 25 ppb, 50 ppb and 100 ppb, respectively (Table I). Similarly, the method precision within-run and between-run was evaluated by calculating coefficient of variation (CV) for different AMP concentrations. In present study, for within-run (intra-assay), the CV was calculated as 6.37% (n=15) while between-run (inter-assay) the CV was 8.98% (n=3). These values were found in acceptable range as described by VICH guidelines.

Table I. Recovery calculation for AMP residues in selected chicken tissues at different spiking levels.

Spiking level	Tissue matrix	Mean O.D	B/Bo	R.A (%)	Final Conc. (ppb)	Re-covery (%)
½ MRL (25 ppb)	Heart	4.269	0.9335	93.35	21.78	87.11
	Liver	4.256	0.9306	93.06	22.63	90.53
	Kidney	4.255	0.9304	93.04	22.70	90.79
	Chest muscle	4.274	0.9345	93.45	21.50	86.01
	Thigh muscle	4.299	0.9401	94.01	20.00	79.98
1 MRL (50 ppb)	Heart	4.049	0.8854	88.54	40.75	81.49
	Liver	4.004	0.8755	87.55	46.38	92.76
	Kidney	4.010	0.8769	87.69	45.53	91.06
	Chest muscle	4.061	0.8880	88.80	39.38	78.75
	Thigh muscle	3.986	0.8715	87.15	48.82	97.64
2 MRL (100 ppb)	Heart	3.773	0.8250	82.50	89.54	89.54
	Liver	3.760	0.8222	82.22	92.78	92.78
	Kidney	3.732	0.8160	81.60	100.62	100.62
	Chest muscle	3.776	0.8256	82.56	88.77	88.77
	Thigh muscle	3.775	0.8254	82.54	89.03	89.03

OD, Optical density; RA, Relative absorbance.

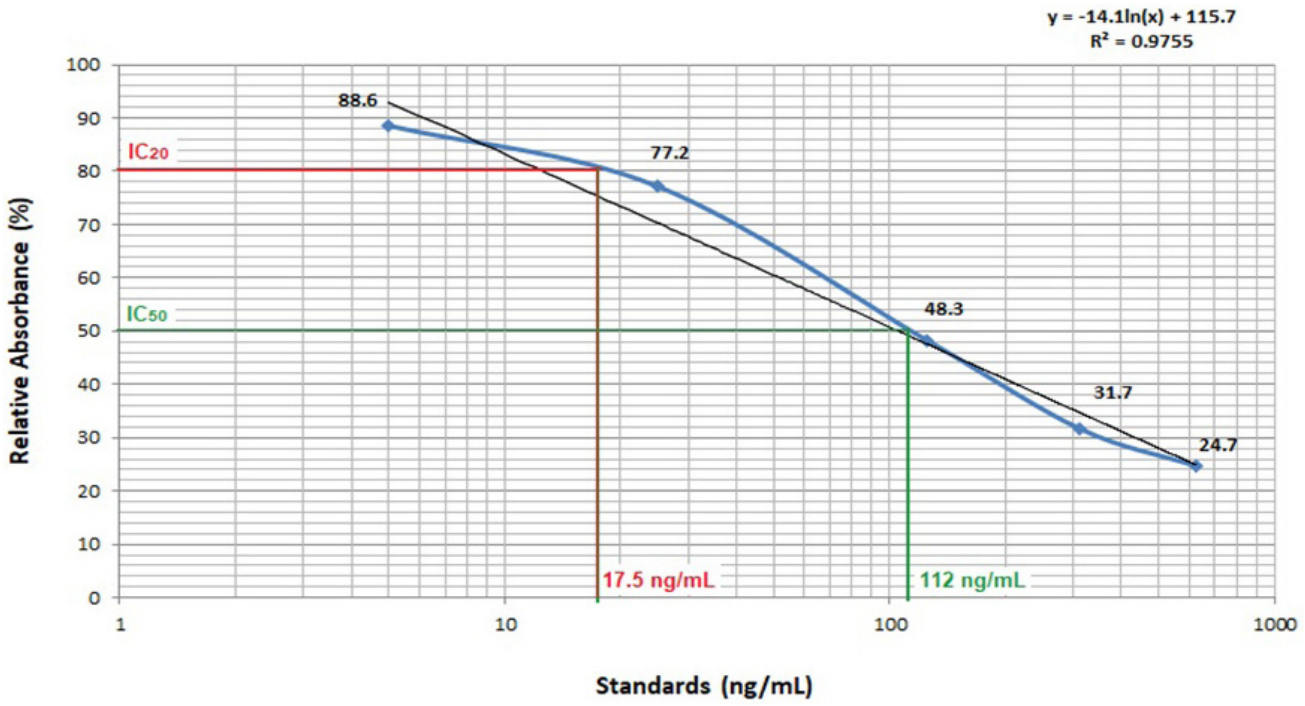

Fig. 2. Calibration curve used to detect Ampicillin residues in different tissues of broiler chicken.

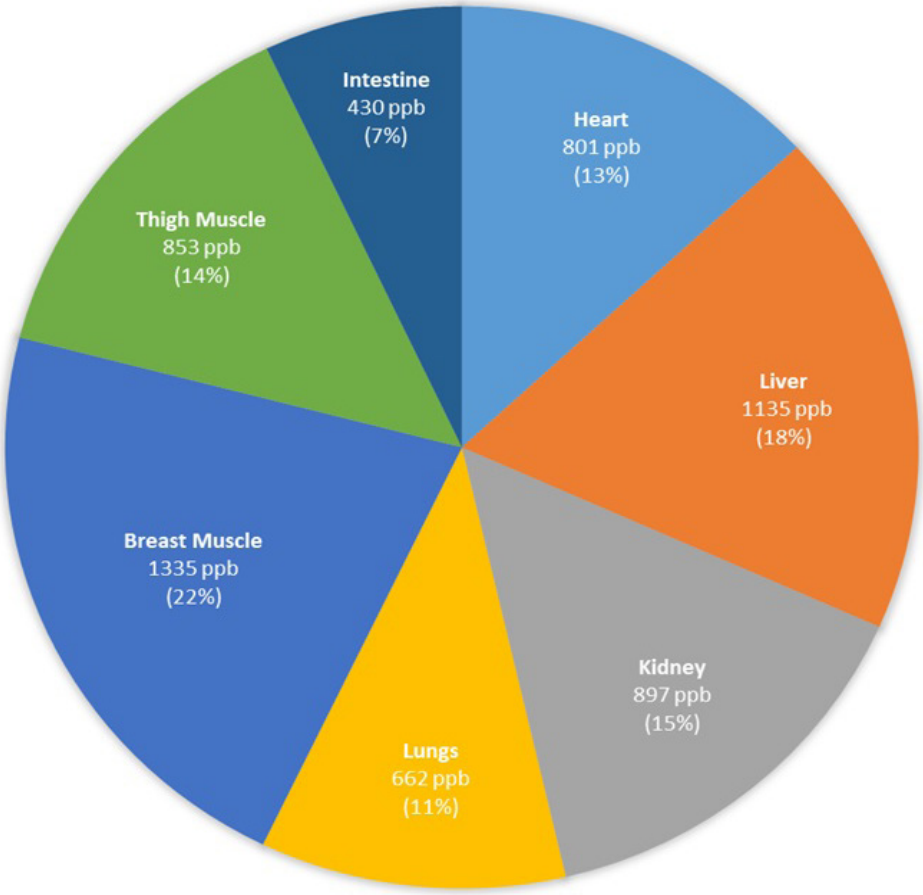

Fig. 3. Bioavailability of Ampicillin in various tissues of broiler chicken after 24 h of dose administration.

Post-administration AMP bioavailability in broiler chickens

After 24 h, the highest AMP residue levels were detected in breast muscle (1335 ppb, 22%), followed by the liver (1135 ppb, 18%) and kidney (897 ppb, 15%), with the lowest residues found in the intestine (430 ppb, 7%) (Fig. 3). This bioavailability trend remained consistent in subsequent sampling up to 168 h.

Tissue-specific AMP depletion in broiler chickens

The results indicated that AMP depletion varied across different tissues. The highest depletion (99.3%) was observed in breast muscles, where AMP concentration decreased from 1335.1 ppb to 9.2 ppb over 168 hours. Conversely, the lowest depletion (98.3%) was recorded in the intestine, with AMP levels reducing from 429.8 ppb to 7.3 ppb within the same period. Overall, AMP depletion (%) showed a gradual increase across all tissues, ranging from 43.3% to 98.9% over a period of 7 days (Table II).

The results indicated that AMP residues gradually decreased over time, with a significant reduction observed up to the 4th day (96 h). However, the concentration remained above the MRL in almost all tissue matrices during this period. In subsequent samplings at 120, 144, and 168 h, the AMP levels further declined, eventually falling below the MRL (Fig. 4).

Occurrence of AMP residues in marketed broiler meat

AMP residues were analysed in 65 broiler meat samples collected from various locations in District Faisalabad. The results indicated that 21.5% of the samples contained detectable AMP residues. Among these, 6 samples (9.23%) exceeded the MRL of 50 ppb, with the highest residue concentration recorded at 101.7 ppb.

Evaluation of health risks

The health risk associated with AMP residues in broiler meat was assessed using the Health Risk Index (HRI). The cut-off value was set at 1, corresponding to

Table II. Mass balance of AMP dose (40 mg/kg) in broiler chickens over a 7-day period.

Parameters	Concentration of AMP (ppb) in treated samples							Overall depletion (%)
	Day 1 (24 h)	Day 2 (48 h)	Day 3 (72 h)	Day 4 (96 h)	Day 5 (120 h)	Day 6 (144 h)	Day 7 (168 h)	
Heart	800.8	652.3	330.3	89.4	39.3	16.2	9.1	98.86
Liver	1135.1	806.5	433.5	194.5	62.2	23.9	11.1	99.02
Kidney	897.4	572.3	474.9	127.2	36.6	16.5	12.3	98.63
Lungs	661.7	437.9	284.4	84.4	27.7	18.5	8.1	98.77
Breast muscle	1335.1	782.8	346.7	113.5	50.6	22.9	9.2	99.31
Thigh muscle	852.6	467.5	187.4	69.5	42.4	17.9	7.4	99.13
Intestine	429.8	363.4	173.3	55.7	26.5	11.8	7.3	98.30
Total Conc.	6112.6	4082.9	2230.8	734.5	285.5	127.9	64.6	98.94
Depletion (%)	-	43.31	73.50	87.98	95.33	97.91	98.94	-

Values are mean of three replicates.

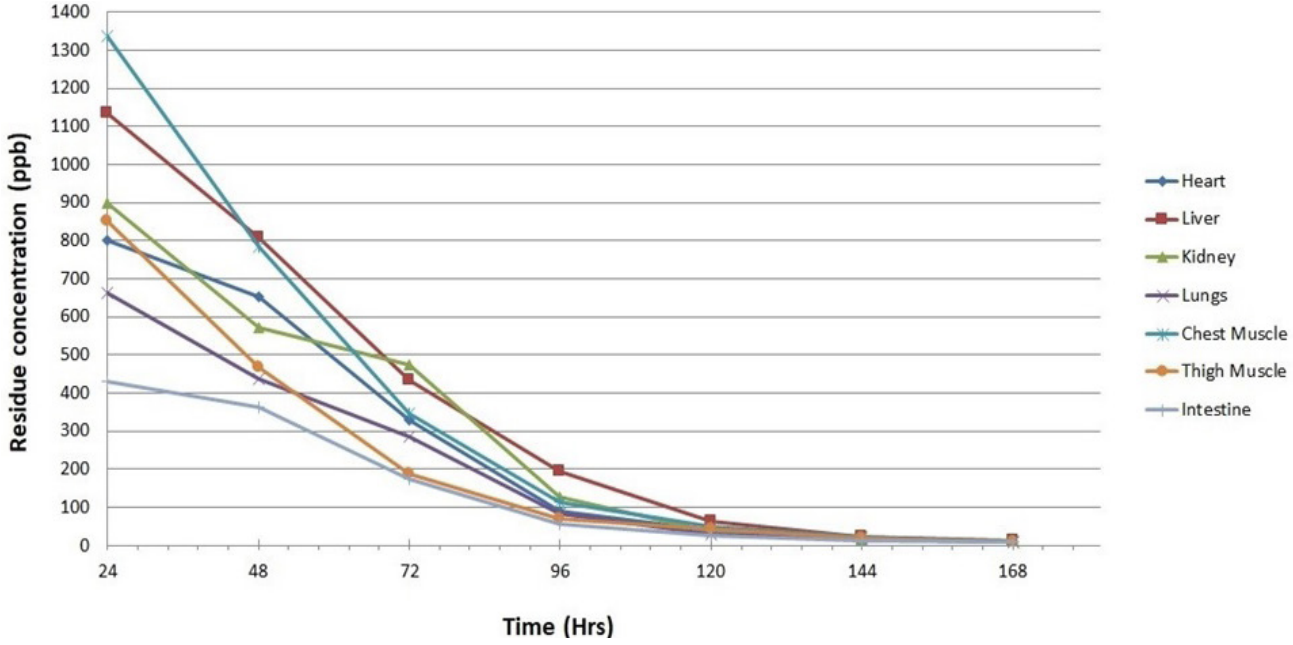

Fig. 4. Depletion trend of Ampicillin residues in various tissues of broiler chicken.

the MRL of 50 ppb. The HRI more than one indicated a high health risk, between zero to 1 was classified as low risk and with no AMP residues was considered safe for consumption. Among 65 tested samples, 6 samples (9.23%) had an HRI exceeding 1, suggesting a potential health risk due to AMP contamination. The detection rate of AMP residues in broiler meat was 21.54%, with 9.23% posing a high health risk, 12.31% classified as low risk, and 78.46% deemed safe for consumption (Table III).

DISCUSSION

The poultry industry is a key player in the global food sector, with chicken being the most widely farmed species. Each year, over 90 billion tons of chicken meat are produced worldwide (FAO, 2017). The worldwide consumption of poultry meat increased from 11 kg per person in 2000 to 14.4 kg in 2011, with projections suggesting it could reach 17.2 kg per capita by 2030 (Anonymous, 2015). Poultry meat serves as a more affordable and nutritionally suitable alternative to mutton and beef. However, the unchecked use of drugs and inadequate biosafety measures for their withdrawal have led to a decline in meat quality (Mund *et al*., 2017). Veterinary consultation prevents improper antibiotic use, reducing risks. Farmer awareness and good farm practices are key to minimizing unnecessary antibiotics (Muaz *et al*., 2018).

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

Sample code	AMP conc. (ppb)	Daily intake (ppb)	Health risk index (HRI)	Health risk
BMT-23-006	11.9	0.0045	0.238	Low
BMT-23-011	35.4	0.0133	0.708	Low
BMT-23-016	88.6	0.0332	1.772	High
BMT-23-021	22.7	0.0085	0.454	Low
BMT-23-029	17.3	0.0065	0.346	Low
BMT-23-033	56.4	0.0212	1.128	High
BMT-24-004	22.5	0.0084	0.45	Low
BMT-24-005	60.8	0.0228	1.216	High
BMT-24-008	134.5	0.0504	2.69	High
BMT-24-014	101.7	0.0381	2.034	High
BMT-24-020	19.2	0.0072	0.384	Low
BMT-24-023	41.8	0.0157	0.836	Low
BMT-24-025	61.4	0.0230	1.228	High
BMT-24-029	22.5	0.0084	0.45	Low

BMT, Broiler meat; Cut-off value for HRI = 1 (set at MRL 50 ppb).

In many developing countries, antibiotics are widely used in poultry farming to enhance productivity (Van Boeckel *et al*., 2015; Boamah *et al*., 2016). Over 60% of the world's antibiotic production is estimated to be used in livestock farming, including poultry (Mulchandani *et al*., 2023). However, many of these antibiotics are also vital for human medicine. Their excessive and unregulated use raises significant health concerns, as residues can accumulate in meat, eggs, and other poultry products, posing potential risks to human health (Mehdizadeh *et al*., 2010; Goetting *et al*., 2011; Darwish *et al*., 2013).

In poultry, orally administered AMP is quickly absorbed, widely distributed in body fluids and tissues and primarily excreted through urine. A broiler study estimated its oral bioavailability at 30% (Sumano-Lopez and Gutierrez-Olivera, 2010). Kandeel (2014) reported oral AMP bioavailability at 55% in healthy 6-days old chicks but significantly lower at 14% in those with coccidiosis. Studying the pharmacokinetics of AMP esters, which have higher bioavailability in humans than AMP after a single oral dose in different animal species could enhance scientific knowledge and literature (Guzelaydin and Yildirim, 2024).

Guzelaydin *et al*. (2025) studied the pharmacokinetics of AMP and its prodrug bacampicillin (BAC) in chickens and turkeys. In chickens, the elimination half-life of intravenous AMP was 1.81 h, while oral AMP and BAC were 3.64 and 5.39 h, respectively. In turkeys, AMP's half-life was similar for both routes, at 2.44 and 2.53 hours. Oral bioavailability was low, at 25.9% in chickens and 19.1% in turkeys. Due to inconsistent absorption and the risk of residues in food products, oral AMP use in veterinary treatment remains limited. Earlier, Fernandez-Varon *et al*. (2006) was reported that AMP exhibited a similar absorption rate when administered orally and intramuscularly (20 mg/kg) in chickens.

Akond *et al*. (2012) reported 88% AMP resistant Salmonella in hand wash, intestinal fluid, cloacal swabs, egg surfaces, and soil samples from a layer farm in Dhaka. A decade later, Sarker *et al*. (2021) found similar resistance in both broilers and layers in Rajshahi. Talukder *et al*. (2021) observed 100% resistance to AMP and AMOX in Chittagong and Mymensingh. Additionally, Salmonella resistance to these antibiotics ranged from 40% to 92.9% in broilers and layers (Haque *et al*., 2021). Sharma *et al*. (2019) reported 95.71% AMP-resistant Salmonella in poultry samples from India.

Hamamoto and Mizuno (2017) analysed AMP residues in the liver, kidney and skin after a 2-day withdrawal in ten male and ten female White Leghorn chickens fed AMP-medicated feed (40 mg/kg body weight per day) for a week. Using LC-MS, mean recoveries ranged from 93% to 103%, with LOQ 0.1-1.4 ng/g. Residue levels were ≤7.82 ng/g in the skin and ≤0.64 ng/g in the kidney, staying below Japan's provisional maximum limits. These findings confirm the method's reliability and the adequacy of the withdrawal period.

Hakem *et al*. (2013) confirmed poultry meat contamination with β-lactam and/or tetracycline (75.8%), macrolide and/or β-lactam (44.3%), sulfonamide (36.3%), and aminoglycoside (13.7%) residues. Zhao *et al*. (2015) conducted a residue depletion study on AMP in laying hens after oral dosing (60 and 120 mg/kg body weight daily for 5 days). Theoretical withdrawal times in whole eggs were 6.73 and 7.30 days, respectively. AMP residues fell below 10 µg/kg within 7-8 days post-withdrawal, suggesting a recommended withdrawal period of 7-8 days for egg production.

CONCLUSION

AMP residues demonstrated a consistent decline over a 96-h (4-day) period; however, concentrations in most tissue matrices remained above the maximum residue limit (MRL) of 50 µg/kg during this time. Further reductions were observed in subsequent samplings, with residue levels falling below the MRL. Based on this depletion pattern, a withdrawal period of 6 days is recommended, as all samples tested after 144 h were within acceptable safety

limits. Monitoring data identified AMP residues exceeding the MRL in 6 out of 65 commercial broiler meat samples. Health risk assessment indicated 9.23% potential risk to consumers from the intake of AMP-contaminated broiler meat. The implementation of stringent management practices and continuous residue monitoring at poultry farms is essential to reduce the likelihood of contamination and ensure food safety.

DECLARATIONS

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IRB approval

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

Ethical approval

This study involved only broiler chickens and did not include human subjects. Animal use was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Sciences Division, NIAB, Faisalabad (Approval No. NIAB/2024-004). 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 data analysis, preparation and writing of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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