

Food safety and public health implications by determining the antibiotic residues in animal-derived food commercially available in southern Punjab, Pakistan

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Abstract

This comprehensive study aimed to estimate the prevalence of antimicrobial residues in beef, mutton, chicken, fish, and milk. Twenty fresh samples of chicken, mutton, beef, fish, and bovine milk were collected aseptically from retail shops in Southern Punjab, Pakistan. The enzyme-linked immunosorbent assay (ELISA) technique was used to screen the samples for various antimicrobial residues. The ELISA screening of fish samples revealed residue contamination with tetracycline, as well as the metabolites 3-amino-2-oxazolidinone (AOZ) and 5-methyl-morpholino-3-amino-2-oxazolidinone (AMOZ), with concentration ranges of 2.00-108 parts per billion (ppb), 0.09-0.47 ppb, and 0.01-1.08 ppb, respectively. Chicken samples showed residues of tetracycline, quinolone, AOZ, and AMOZ, with detected residual concentrations ranging from 5.60 to 126 ppb for tetracycline, 18 to 230 ppb for quinolone, 0.20 to 1.96 ppb for AOZ, and 0.01 to 1.02 ppb for AMOZ. In livestock, milk samples exhibited 100% contamination for penicillin and 10% for chloramphenicol residues, with concentrations of 0.2-4.1 ppb and 0.01-0.19 ppb, respectively. Beef samples tested positive for residues of penicillin (20%), tetracycline (40%), and chloramphenicol (45%), with residual ranges of 1.36-88 ppb for penicillin, 3.7-148 ppb for tetracycline, and 0.04-0.37 ppb for chloramphenicol. Mutton samples were contaminated with 10% tetracycline and 25% quinolones, showing residual ranges of 1.28-108 ppb for tetracycline and 34-520 ppb for quinolones. Out of 100 samples, 23 showed residues exceeding the maximum residue limit. Specifically, 30.4% of chicken samples, 17.3% of mutton samples, 30.4% of beef samples, 17.3% of milk samples, and 4.3% of fish samples were affected. This study conclusively demonstrated the presence of various antibiotic residues in food-producing animal samples.

Introduction

Food is essential for human growth, proper functioning, and survival. On average, a person consumes 30 tons of food in their lifetime (Rattan and Kaur, 2022). According to the World Health Organization (WHO), an estimated 600 million people, or almost 1 in 10, become ill after consuming contaminated food. This

results in the loss of 42,000 lives annually, emphasizing the inter-link between food safety and security (Lee and Yoon, 2021; WHO, 2022). Antimicrobials are commonly used in the livestock industry to treat and prevent diseases (Hosain *et al.*, 2021). However, weak regulations and poor management practices have led to the excessive use of antimicrobials in food-producing animals (Berman *et al.*, 2023). Globally, 99,502 tons of antimicrobials were used in 2020, and usage is projected to increase by 8% to 107,472 tons by 2030, based on recent antimicrobial trends (Mulchandani *et al.*, 2023).

Pakistan is a lower and middle-income country with a rising demand for protein-based food, including milk and meat (Sohaib and Jamil, 2017). Only in the chicken farms, 462.57 mg/PCU antimicrobials are used, and 60% of these antibiotics used in broiler chicken were critically important antimicrobial (CIA) classes for human medicine as distinguished by the WHO (Umair *et al.*, 2021). According to a study conducted in Pakistan, antimicrobial uses in dairy farms were 138.34 kg, 65.88 mg/kg, and 47.71 DDDA/1000 cow-days; 42.6% of all the antimicrobials used in dairy farms were listed in the CIA for human medicine (Umair *et al.*, 2020). Excessive usage of antimicrobials in livestock selects the resistant bacteria and genetic determinants that may be transmitted to humans through direct contact with animals, the food chain, or the environment (Pandey *et al.*, 2024), resulting in increasing the dependency on antibiotics and the resultant antimicrobial residues in the products yielded for human utilization (Okocha *et al.*, 2018). These residues in the food chain cause the development of drug resistance, disruption of normal intestinal flora, carcinogenicity (sulfamethazine, oxytetracycline, and furazolidone), mutagenicity, nephropathy (gentamicin), hepatotoxicity, reproductive disorders, bone marrow toxicity (chloramphenicol), and even anaphylactic shock in humans (Treiber and Beranek-Knauer, 2021; Arsène *et al.*, 2022). Maximum residue limits (MRLs) are the highest levels of residues that are legally allowed in food products from animals treated with veterinary medicines. Tetracyclines, quinolones, and penicillin are commonly used in food-producing animals (Granados-Chinchilla and Rodríguez, 2017). These antimicrobials' MRL is 100 parts per billion (ppb); penicillin for milk is 4 ppb, and for tissue is 50 ppb (Codex Alimentarius, 2023). Nitrofurans and chloramphenicol are banned drugs in animals, but are still being used in livestock (Schmerold *et al.*, 2023). There are no European Union (EU) MRLs for the banned substances; in 2003, the European Commission defined the minimum required performance limit (MRPL) for chloramphenicol as 0.3 µg per kg, which is the minimum acceptable sensitivity of a test for chloramphenicol residues for all the food matrices, including meat and milk (Codex Alimentarius, 2023). In Pakistan, there is limited data available on the prevalence of used and banned drug residues in the animal food chain. Recently, a study was conducted in Pakistan on the presence of tetracycline residue and tetracycline-resistant gene (*tet*) in food-producing animals (Qamar *et al.*, 2023). Therefore, the present study aimed to identify the antimicrobial residues of quinolones, nitrofurans metabolites, tetracyclines, chloramphenicol, and penicillin in raw milk, mutton, beef, chicken, and fish. Further, it discusses the significance of bacterial food contamination for the "One Health" perspective and food safety and security in the 21st century.

Materials and Methods

Ethical approval

The ethical review committee at the Nuclear Institute for Agriculture and Biology, Faisalabad, approved the current research. Notably, all animal experimentation was conducted by applicable laws, regulations, and guidelines, prioritizing animal welfare and minimizing potential harm.

Animal food samples collection

This is a cross-sectional study to determine the prevalence of antibiotic residues in food samples. A total of 20 beef, 20 mutton, 20 chicken, 20 fish, and 20 milk samples were collected from three different Dera Ghazi Khan (DG Khan) metropolitan areas. DG Khan is in the southwestern part of Punjab, the 19th biggest city by population, and it lies west of the Indus River (Figure 1). 100-150 g of meat samples (beef, mutton, fish, and chicken) were collected in sterile zip-lock bags, stored in an icebox at 4°C, and transported to the research laboratory within one hour. 50-70 mL of raw milk samples were collected in sterile conical tubes from raw milk shops. These samples were stored in an icebox at 4-6°C and transported to the research laboratory within one hour for further analysis. The graphic workflow of the methodology for the detection of antimicrobial residues in different animal food samples is given in Figure 2.

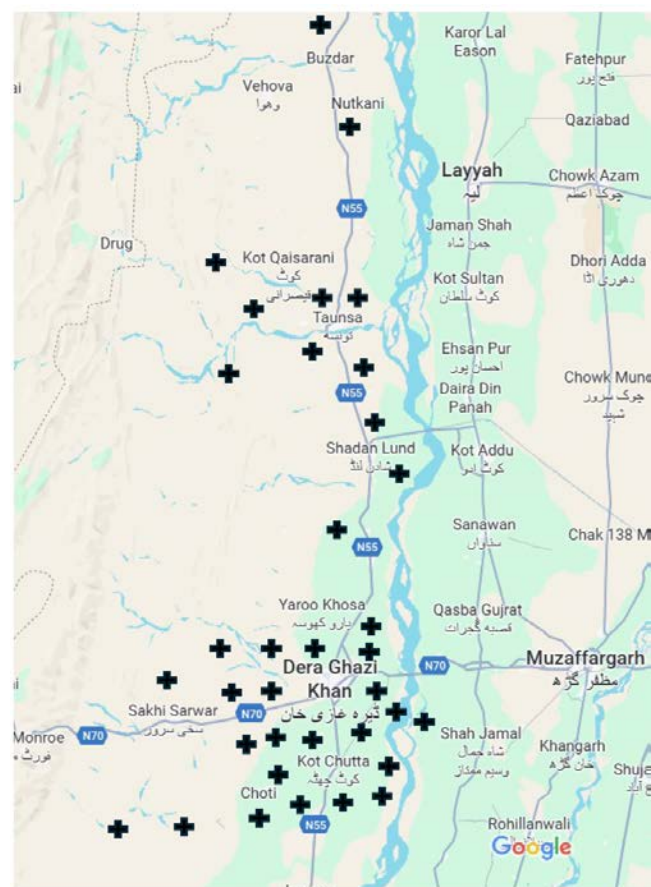


Figure 1. Black crosses showing the sample collection sites in southern Punjab across the Indus River, Pakistan.

Detection of the antimicrobial residue of penicillin in beef tissue and bovine milk samples

A commercially available enzyme-linked immunosorbent assay (ELISA) (Euro Proxima, Biopharm, Darmstadt, Germany) kit was used for penicillin residues in beef and milk.

For beef residue detection, 1 g of beef samples was minced, homogenized, and diluted in 50 mL conical tubes. After homogenization, samples were centrifuged at room temperature for 10 minutes at 2000 rpm. 50 μ L of supernatant was collected into the new glass vial and again diluted with dilution buffer. 50 μ L was used for assay development.

For milk, 1 mL of milk sample was taken in a conical tube and vortexed for 3 seconds; 250 μ L of milk was collected in glass tubes and diluted with 250 μ L of dilution buffer, again vortexed for 3 seconds. 50 μ L was used for assay development. The absorbance was measured at 450 nm *via* an ELISA plate reader (BioTek, Burbank, California, USA). The residue concentration was calculated using Eq. 1 mentioned in the manual.

$$\frac{\text{Absorbance samples/standard}}{\text{Absorbance zero standards}} \times 100$$

[Eq.1]

Detection of the tetracycline residue in chicken, mutton, beef, and fish tissue samples

ELISA (RANDOX, Crumlin Co., Antrim, UK) detected tetracycline residues in beef, chicken, fish, and mutton samples. In

short, 1 g of the meat samples was minced, homogenized, and diluted in a 50 mL conical tube. After shaking, samples were centrifuged for 10 minutes at room temperature, and 200 μ L of supernatant was collected into a new glass vial and diluted with wash buffer. 50 μ L was used for assay development. The absorbance was measured at 450 nm *via* an ELISA plate reader (BioTek, Burbank, California, USA). The residue concentration was calculated using Eq. 1.

Detection of quinolone residues in chicken and mutton tissue samples

Quinolone residues were detected in chicken and mutton *via* ELISA (Quicking Biotech, Shanghai, China). In short, 1 g of the meat samples was minced and homogenized, and 70 % methanol was added to a 50 mL conical tube. After shaking, samples were centrifuged for 10 minutes at 5000 rcf at room temperature. 100 μ L of supernatant was collected into the new glass vial and diluted with 100 μ L of distilled water. 50 μ L was used for assay development. The absorbance was measured at 450 nm *via* an ELISA plate reader (BioTek, Burbank, California, USA). The residue concentration was calculated using Eq. 1.

Detection of chloramphenicol residues (banned drug) in beef tissue and bovine milk samples

The antimicrobial residue of chloramphenicol was detected in beef and milk samples *via* ELISA (Quicking Biotech, Shanghai, China). In brief, 2 mL of milk and 2 g of chopped beef samples were taken in conical tubes. After adding ethyl acetate, samples

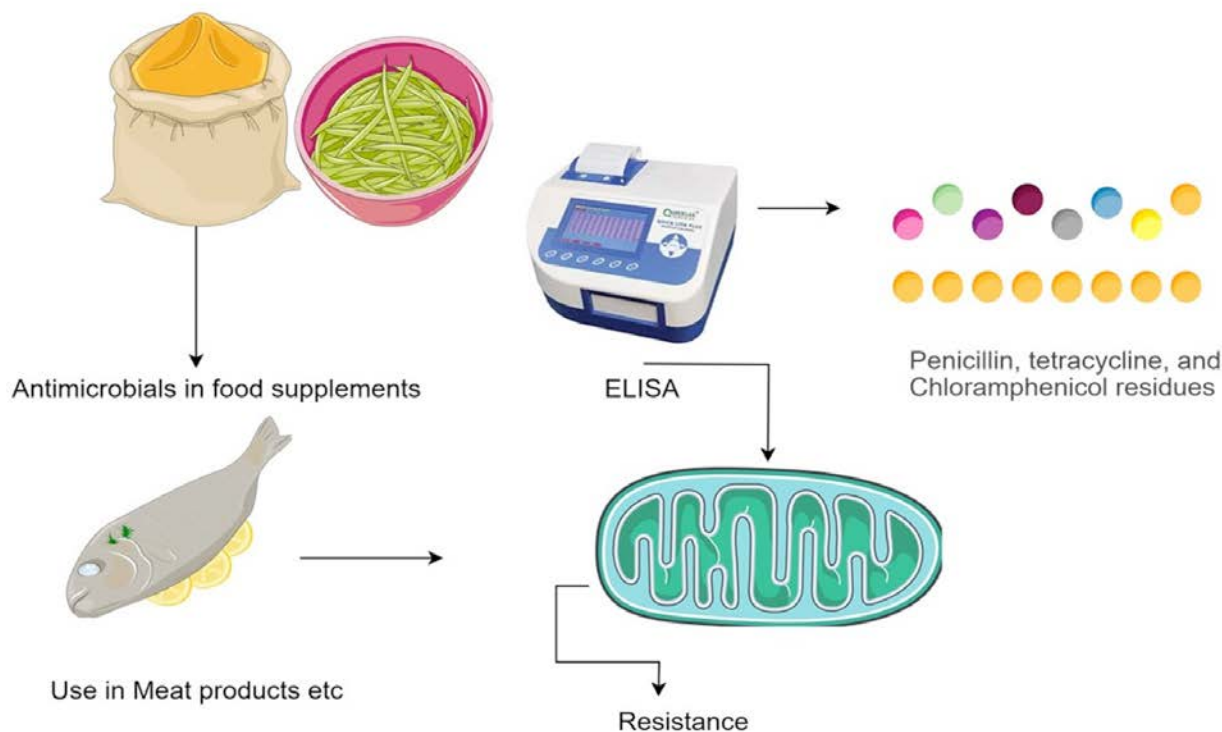


Figure 2. Graphic workflow of the methodology for the prevalence of antimicrobial residues in different animal food samples. ELISA, enzyme-linked immunosorbent assay.

were homogenized for 10 minutes and then centrifuged for 10 minutes at 4000 rcf. After collecting the supernatant in a new glass vial, it was dried by nitrogen flow at 50 °C. Dried samples were diluted and centrifuged again for 10 minutes, and 80 µL of infranatant was collected in the new glass vial and then used for assay development. The absorbance was measured at 450 nm *via* an ELISA plate reader (BioTek, Burbank, California, USA). The residue concentration was calculated using Eq. 1.

Detection of nitrofurantolone metabolite (banned drug) in chicken and fish tissue samples

The ELISA detected the antimicrobial residue of nitrofurantolone in chicken and fish samples (Quicking Biotech, Shanghai, China). 1 g of a defrosted, properly chopped tissue sample was taken in conical tubes. After adding the distilled water, the samples were homogenized for 30 seconds. 1 M HCl was added to all the meat samples, vortexed for 1 minute, and incubated in the dark at 37°C for 16 hours. After incubation, buffer solution, NaOH, and ethyl acetate were added to all samples, mixed, and centrifuged for 10 minutes at room temperature. The supernatant was collected into a new glass vial and then dried by nitrogen flow at 50°C. In the dried samples, hexane was added, diluted with wash buffer, and vortexed. 50 µL of infranatant was collected in the new glass vial and then used for assay development. The absorbance was measured at 450 nm *via* an ELISA plate reader (BioTek, Burbank, California, USA). The residue concentration was calculated using Eq. 1.

Results

Penicillin residue in beef tissue and bovine milk samples

A commercial ELISA kit was used to determine the detection of penicillin residues in bovine milk and beef samples. The kits were standardized and validated using standards, including 0, 0.125, 0.25, 0.5, 1, 4 ppb, and positive and negative controls. To calculate the relative absorbance, optical density was measured at 450 nm, and relative absorbance was used to construct the calibration curve. From the calibration curve, inhibitory concentration 20 (IC₂₀) was calculated at 0.08 ppb, and inhibitory concentration 50 (IC₅₀) at 0.35 ppb. The calibration curve was interpolated to calculate the residue concentration in an unknown sample (Figure 3 A).

The study analyzed 40 samples (20 bovine milk and beef tissue) from District Gera Ghazi Khan in Punjab, Pakistan. The results showed that three bovine milk samples and two beef muscles had positive traces of penicillin residues. The maximum concentration of residues found ranged from 5.2 ppb in milk to 88 ppb in tissue. These levels exceeded the EU MRL limits of 4 ppb for milk and 50 ppb for tissue (*Supplementary Table 1* and Figure 3 B).

Tetracycline residues in chicken and mutton samples

Our research on tetracycline residue detection in meat samples was conducted using a commercial ELISA kit. This kit was not only standardized but also validated using a variety of standards, including positive and negative controls. The optical density was measured at 450 nm to calculate the relative absorbance, which was then used to construct the calibration curve. We calculated IC₂₀ and IC₅₀ from this curve at 0.06 ppb and 0.30 ppb, respectively. The residue concentration in an unknown sample was determined by interpolating the calibration curve (Figure 4A). Our findings from 80 samples (20 of each chicken, mutton, fish, and beef tissue) collected from District Gera Ghazi Khan (Punjab), Pakistan, were as follows. All fish samples were negative, with a maximum concentration of 5.6 ppb. However, two chicken, one mutton, and three beef samples were positive, with maximum concentrations of 126 ppb, 108 ppb, and 148 ppb, respectively. It is important to note that the EU MRL of tetracycline for muscles is 100 ppb (*Supplementary Tables 2 and 3* and Figure 4B and C).

Chloramphenicol (banned drug) residues in milk and beef samples

Chloramphenicol is strictly prohibited in animals that produce food, as it is a banned drug. A commercial ELISA kit was used to determine the detection of chloramphenicol residues in bovine milk and beef samples. The kits were standardized and validated using standards, including 0, 0.025, 0.1, 0.2, 0.4, 1.6 ppb, and positive and negative controls. To calculate the relative absorbance, optical density was measured at 450 nm, and relative absorbance was used to construct the calibration curve. From the calibration curve, IC₂₀ was calculated at 0.08 ppb and IC₅₀ at 0.35 ppb. The calibration curve was interpolated to calculate the residue concentration in an unknown sample (Figure 5A). Results indicated that one milk sample was positive out of 40 samples (20 of each bovine milk and beef tissue) collected from District Gera Ghazi Khan (Punjab), Pakistan,

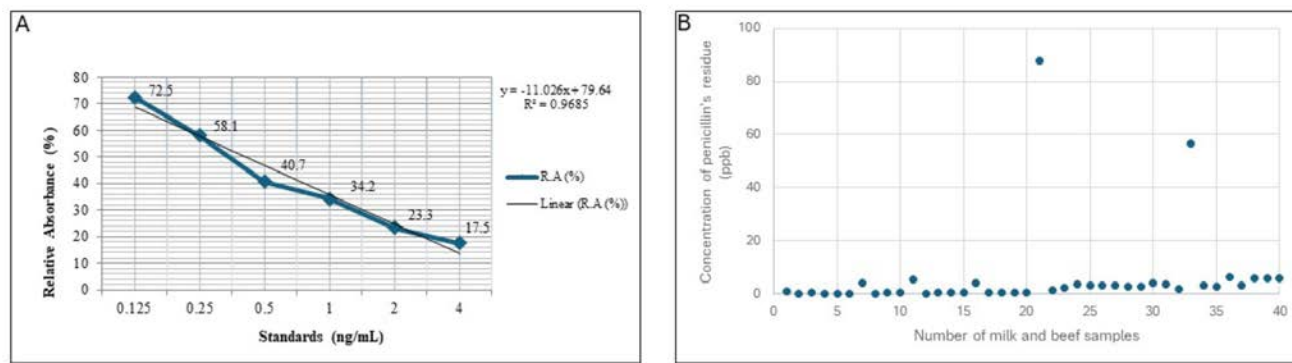


Figure 3. A) Calibration curve for penicillin detection by enzyme-linked immunosorbent assay; B) penicillin residue in beef and milk samples. Blue dots show three bovine milk samples and two beef muscles that had positive traces of penicillin residues.

with a maximum concentration of 0.19 ppb. In comparison, two beef samples have more than a reference point of action (*i.e.*, 0.15 ppb) with a maximum concentration of 0.22 ppb. The EU MRPL for all food matrices is 0.3 ppb (*Supplementary Table 4* and *Figure 5B*).

Quinolones residue in chicken and mutton tissue

A commercial ELISA kit detected quinolone residues in chicken and mutton samples. The kits were standardized and validated using a range of standards, including 0, 1, 3, 9, 27, and 81 ppb, as well as positive and negative controls. Optical density was measured at 450 nm to determine the relative absorbance, and the calibration curve was constructed using the relative absorbance. The IC_{20} and IC_{50} were calculated from the calibration curve at 3.5 ppb and 18 ppb, respectively. The residue concentration in an unknown sample was determined by interpolating the calibration curve (*Figure 6A*). A total of 40 samples (20 each of chicken and mutton tissue) were collected from District Gera Ghazi Khan (Punjab), Pakistan, and analyzed. The results showed that two chicken samples had an MRL of 230 ppb, and three mutton samples had an MRL of 520 ppb, exceeding the EU MRL of quinolones for muscles, which is 100 ppb (*Supplementary Table 5* and *Figure 6B*).

Nitrofuran metabolite (furazolidone-3-amino-2-oxazolidinone) (banned drug)

Furazolidone-3-amino-2-oxazolidinone (AOZ) is prohibited in food-producing animals and is not tolerated under any circum-

stances. A commercial ELISA kit was utilized to detect residues of this substance in fish and chicken samples. The kit was standardized and verified using various standards, including 0, 0.025, 0.05, 0.1, 0.2, 0.5 ppb, and positive and negative controls. The optical density was measured at 450 nm to obtain the relative absorbance, which was used to create the calibration curve. The IC_{20} value was determined to be 0.027 ppb, while the IC_{50} was 0.157 ppb. The calibration curve was then used to calculate residue concentration in an unknown sample (*Figure 7A*). A total of 40 samples (20 fish and 20 chicken tissues) from District Gera Ghazi Khan (Punjab), Pakistan, were analyzed, and all fish samples tested negative for residues, with a maximum concentration of 0.2 ppb. However, two chicken samples had more than half the MRPL value, with a maximum concentration of 1.96 ppb. It is worth noting that the EU MRPL for all food matrices is 1.0 ppb (*Supplementary Table 6* and *Figure 7B*).

Nitrofuran metabolite (banned drug) in fish and beef muscle

Furaltadone-5-morpholinomethyl-3-amino-2-oxazolidone (AMOZ) is also a banned drug in food-producing animals with zero tolerance. A commercial ELISA kit was used to determine the detection of residues in fish and chicken samples. The kits were standardized and validated using standards, including 0, 0.05, 0.1, 0.25, 0.5, 1.0 ppb, and positive and negative controls. To calculate the relative absorbance, optical density was measured at 450 nm,

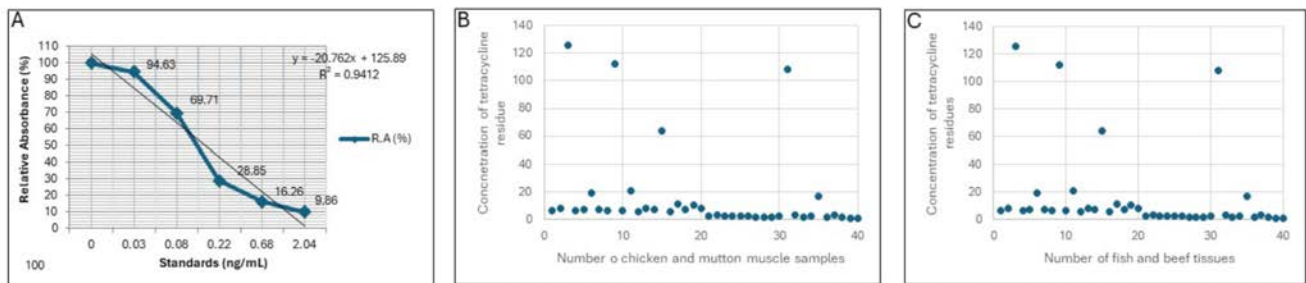


Figure 4. A) Calibration curve for tetracycline detection by enzyme-linked immunosorbent assay; B) the blue dots show the tetracycline residue in chicken and mutton samples; C) the blue dots show tetracycline residue in fish and beef samples.

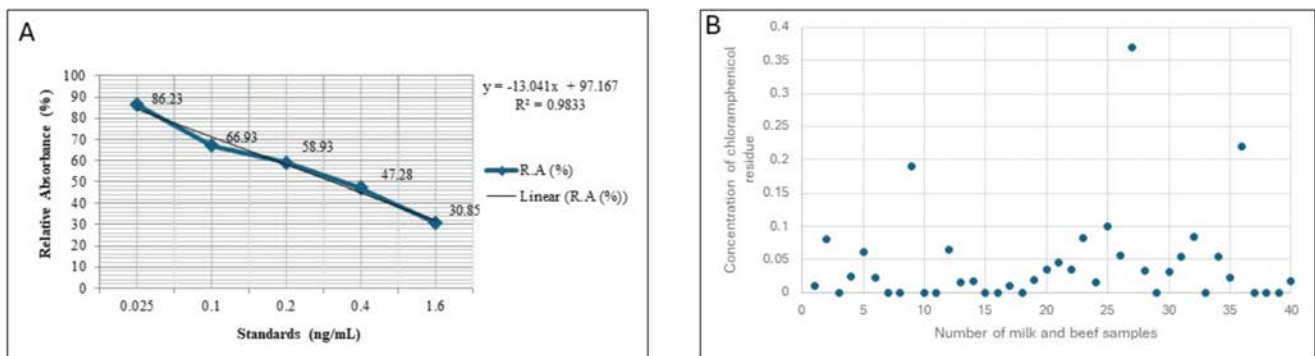


Figure 5. A) Calibration curve for chloramphenicol detection by enzyme-linked immunosorbent assay; B) the blue dots show the chloramphenicol residue in milk and beef samples.

and relative absorbance was used to construct the calibration curve. From the calibration curve, IC_{20} was calculated at 0.04 ppb and IC_{50} at 0.15 ppb. The calibration curve was interpolated to calculate the residue concentration in an unknown sample (Figure 8A). Out of 40 samples (20 of each fish and chicken tissue) collected from District Gera Ghazi Khan (Punjab), Pakistan, were ana-

lyzed. Results indicated that one fish and one beef sample were positive for AMOZ metabolite with a maximum concentration of 1.08 ppb and 1.02 ppb, respectively. The EU MRPL for all food matrices is 1.0 ppb (*Supplementary Table 7* and Figure 8B). Further, *Supplementary Table 8* displays an overview of all the antibiotic residues in different food samples.

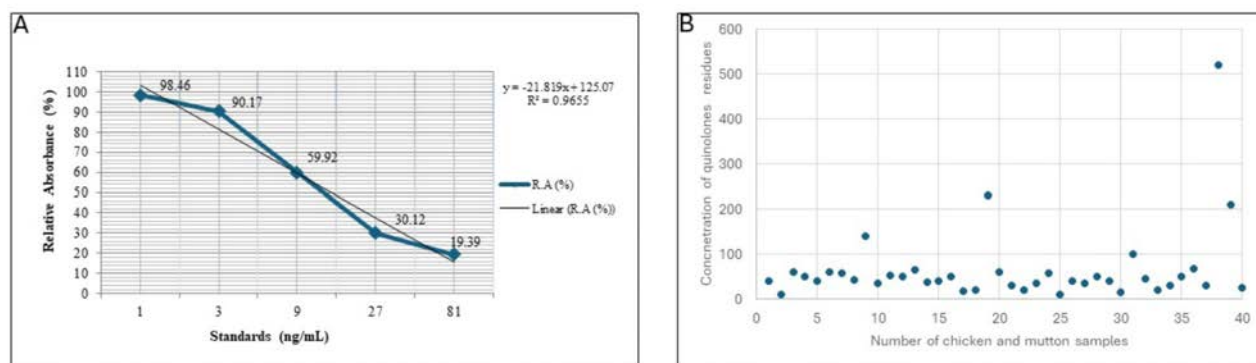


Figure 6 A) Calibration curve for quinolones detection by enzyme-linked immunosorbent assay; B) the blue dots show quinolones in chicken and mutton tissues.

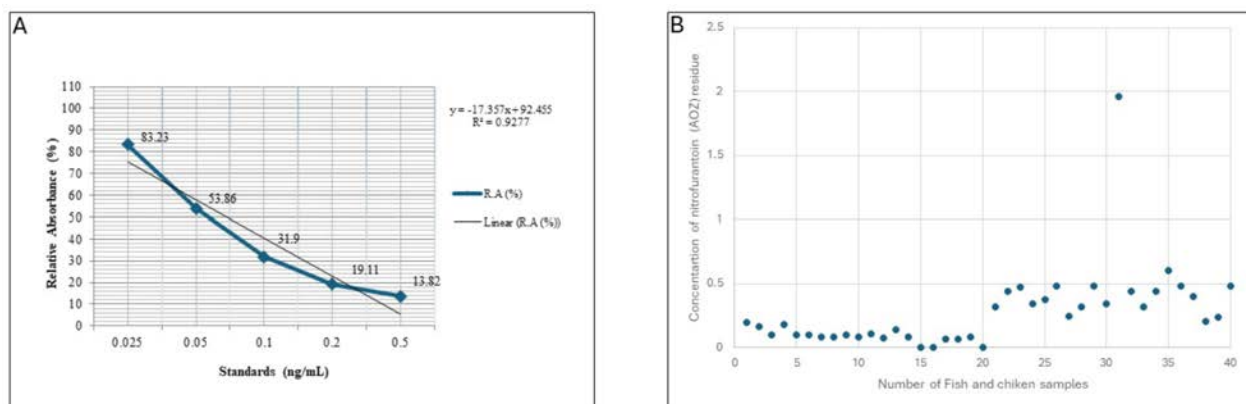


Figure 7. A) Calibration curve for nitrofurans metabolite [3-amino-2-oxazolidinone (AOZ)] detection by enzyme-linked immunosorbent assay; B) the blue dots show the nitrofurans metabolite (AOZ) in fish and chicken meat.

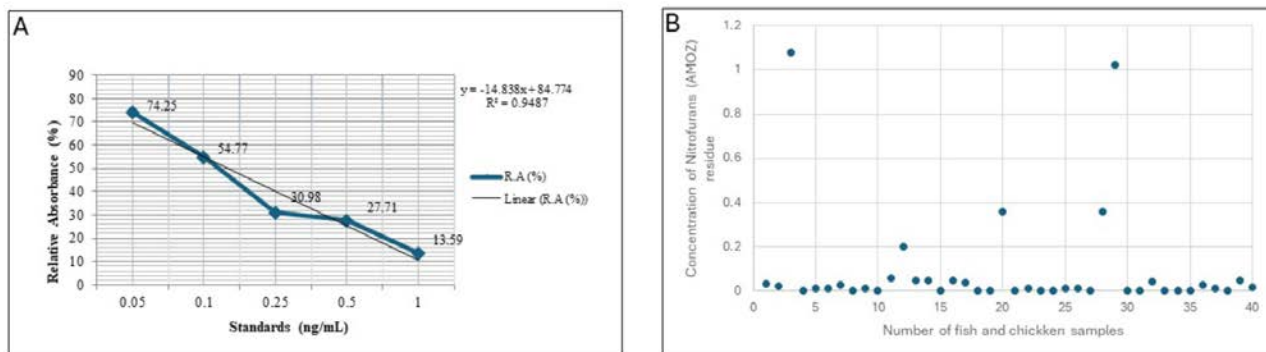


Figure 8. A) Calibration curve for nitrofurans metabolite [5-morpholinomethyl-3-amino-2-oxazolidone (AMOZ)] detection by enzyme-linked immunosorbent assay; B) the blue dots show nitrofurans metabolite (AMOZ) residue in fish and chicken samples.

Discussion

Food safety is a significant public health issue; more than 200 diseases, from diarrhea to cancer, are caused by unsafe food containing harmful microbes (Gizaw, 2019). According to the WHO, over 40% of children under 5 fall ill after consuming contaminated food, with 125,000 deaths annually (Faour-Klingbeil and Todd, 2019). Global protein demand increased by 40 % from 2000-2018 and is directly linked to the increasing demand for poultry meat consumption, and by 2031, poultry meat consumption is predicted to reach 153.85 metric kilotons. Food safety is a pressing issue in developing countries, as they endure most of the burden of food-borne diseases (Henchion *et al.*, 2017). In this study, five different antibiotics (tetracycline, chloramphenicol, penicillin, quinolone, and nitrofurantoin metabolites (AOZ and AMOZ) residues in animal food products were detected, and 100 samples were analyzed *via* ELISA for residual detection. According to this study, the prevalence of antimicrobial residues of tetracycline in 80 meat samples (20 each of chicken, fish, mutton, and beef tissue) is relatively low. The findings show that two chicken, one mutton, and three beef samples have more than 100 ppb tetracycline residues. In contrast, all fish samples have tetracycline residue levels below 100 ppb. The MRL of tetracycline in meat is 100 µg per kg, according to the EU and Codex Alimentarius Commission. A study conducted in Tanzania also found tetracycline residue in meat samples, but their residue levels were more than the MRLs (Ulomi *et al.*, 2022). Another study in Faisalabad by Qamar *et al.* detected tetracycline residue in meat and milk samples. Still, they also detected antimicrobial-resistant bacteria and resistance genes in milk and meat (Qamar *et al.*, 2023). For penicillin residues, according to the results, three milk samples and two beef samples have more penicillin residues than the EU MRL. The MRL for penicillin in milk is 4 ppb; in tissue, it is 50 ppb. These results were similar to the study conducted in Lebanon for penicillin residue detection in milk and dairy products; all the samples were within the reference range (Kabrite *et al.*, 2019). A study conducted in Iran also detected penicillin residues in milk samples *via* the ELISA colorimetric method (Kabrite *et al.*, 2019). In quinolones residue detection, two chicken samples and three mutton samples contain more than 100 ppb residue, and the EU MRL for quinolones is 100 ppb. A study conducted in Indonesia detected fluoroquinolones in chicken meat, but their residual values were considered safe for dietary intake (Widiastuti *et al.*, 2022). A recent study conducted in China also detected the antimicrobial residues of quinolones in poultry food *via* the liquid chromatography/mass spectrometry method (Yang *et al.*, 2020). Chloramphenicol is a banned drug in food-producing animals due to its carcinogenic effects; in this study, one milk sample and two beef samples contained residues with more than the reference point of action (*i.e.*, 0.15 ppb). The EU MRPL for all food matrices is 0.3 ppb. A study conducted in Turkey also detected chloramphenicol residues in milk *via* high-performance liquid chromatography with diode array detection (Vuran *et al.*, 2021). Nitrofurantoin is also a banned drug, and in the present study, two metabolites, AOZ and AMOZ, were detected in fish and chicken samples; the results showed that two chicken samples for AOZ metabolites and only one chicken and one fish sample for AMOZ showed the residue more than the MRPL value. In the present study, four samples of fish (20%) and two samples of chicken (10%) contained AMOZ residues, but all were within the MRPL value. A study conducted in France detected the five nitrofurantoin metabolites in meat and aquaculture products *via* the LC-MS/MS method (Guichard *et al.*, 2021). This study has a few limitations,

including other drugs' antimicrobial residues and antimicrobial-resistant genes.

Conclusions

This study found antibiotic residues in various food samples. Tetracycline and quinolone residues were primarily detected in beef and chicken, with increased MRL. Additionally, some banned drugs, such as chloramphenicol and nitrofurantoin metabolites, were also identified with elevated MRLs. The study emphasizes the importance of minimizing antibiotic use and adhering to withdrawal times to reduce the risks associated with antimicrobial residues in animal products, which can lead to serious consequences. Consequently, the "One Health" approach is presented as the optimal solution to emerging global health issues.

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Online supplementary material:

Supplementary Table 1. Determination of penicillin residues in milk and beef samples.

Supplementary Table 2. Determination of tetracycline in chicken and mutton muscles.

Supplementary Table 3. Determination of tetracycline in fish and beef tissues.

Supplementary Table 4. Determination of chloramphenicol residues in bovine milk and beef samples.

Supplementary Table 5. Determination of quinolones in chicken and mutton tissue.

Supplementary Table 6. Determination of nitrofurans metabolite (3-amino-2-oxazolidinone) in fish and chicken meat.

Supplementary Table 7. Determination of nitrofurans metabolite (5-morpholinomethyl-3-amino-2-oxazolidone) in Fish and chicken meat.

Supplementary Table 8. Antibiotic residues in different food matrices.